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(57) Abstract

Targetable diagnostic and/or therapeutically active agents, e.g. ultrasound contrast agents, comprising a suspension in an aqueous carrier liquid of a reporter comprising gas–containing or gas–generated material, in which the reporter is coupled or linked to one or more non–bioactive vectors.

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Improvements in or relating to diagnostic/therapeutic agents

This invention relates to diagnostic and/or therapeutically active agents, more particularly to diagnostic and/or therapeutically active agents incorporating moieties which interact with or have affinity for sites and/or structures within the body so that diagnostic imaging and/or therapy of particular locations within the body may be enhanced. Of particular interest are diagnostic agents for use in ultrasound imaging, which are hereinafter referred to as targeted ultrasound contrast agents.

It is well known that ultrasound imaging comprises a potentially valuable diagnostic tool, for example in studies of the vascular system, particularly in cardiography, and of tissue microvasculature. A variety of contrast agents has been proposed to enhance the acoustic images so obtained, including suspensions of solid particles, emulsified liquid droplets, gas bubbles and encapsulated gases or liquids. It is generally accepted that low density contrast agents which are easily compressible are particularly efficient in terms of the acoustic backscatter they generate, and considerable interest has therefore been shown in the preparation of gas-containing and gas-generating systems.

Gas-containing contrast media are also known to be effective in magnetic resonance (MR) imaging, e.g. as susceptibility contrast agents which will act to reduce MR signal intensity. Oxygen-containing contrast media also represent potentially useful paramagnetic MR contrast agents.

Furthermore, in the field of X-ray imaging it has been observed that gases such as carbon dioxide may be used as negative oral contrast agents or intravascular

contrast agents.

The use of radioactive gases, e.g. radioactive isotopes of inert gases such as xenon, has also been proposed in scintigraphy, for example for blood pool imaging.

Targeted ultrasound contrast agents may be regarded as comprising (i) a reporter moiety capable of interacting with ultrasound irradiation to generate a detectable signal; (ii) one or more vectors having affinity for particular target sites and/or structures within the body, e.g. for specific cells or areas of pathology; and (iii) one or more linkers connecting said reporter and vector(s), in the event that these are not directly joined.

The molecules and/or structure to which the agent is intended to bind will hereinafter be referred to as the target. In order to obtain specific imaging of or a therapeutic effect at a selected region/structure in the body the target must be present and available in this region/structure. Ideally it will be expressed only in the region of interest, but usually will also be present at other locations in the body, creating possible background problems. The target may either be a defined molecular species (i.e. a target molecule) or an unknown molecule or more complex structure (i.e. a target structure) which is present in the area to be imaged and/or treated, and is able to bind specifically or selectively to a given vector molecule.

The vector is attached or linked to the reporter moiety in order to bind these moieties to the region/structure to be imaged and/or treated. The vector may bind specifically to a chosen target, or it may bind only selectively, having affinty also for a limited number of other molecules/structures, again creating possible background problems.

There is a limited body of prior art relating to targeted ultrasound contrast agents. Thus, for example,

US-A-5531980 is directed to systems in which the reporter comprises an aqueous suspension of air or gas microbubbles stabilised by one or more film-forming surfactants present at least partially in lamellar or laminar form, said surfactant(s) being bound to one or more vectors comprising "bioactive species designed for specific targeting purposes". It is stated that the microbubbles are not directly encapsulated by surfactant material but rather that this is incorporated in liquid-filled liposomes which stabilise the microbubbles. It will be appreciated that lamellar or laminar surfactant material such as phospholipids present in such liposomes will inevitably be present in the form of one or more lipid bilayers with the lipophilic tails "back-to-back" and the hydrophilic heads both inside and outside (see e.g. Schneider, M. on "Liposomes as drug carriers: 10 years of research" in *Drug targeting, Nyon, Switzerland, 3-5 October 1984*, Buri, P. and Gumma, A. (Ed), Elsevier, Amsterdam 1984).

EP-A-0727225 describes targeted ultrasound contrast agents in which the reporter comprises a chemical having a sufficient vapour pressure such that a proportion of it is a gas at the body temperature of the subject. This chemical is associated with a surfactant or albumin carrier which includes a protein-, peptide- or carbohydrate-based cell adhesion molecule ligand as vector. The reporter moieties in such contrast agents correspond to the phase shift colloid systems described in WO-A-9416739; it is now recognised that administration of such phase shift colloids may lead to generation of microbubbles which grow uncontrollably, possibly to the extent where they cause potentially dangerous embolisation of, for example, the myocardial vasculature and brain (see e.g. Schwarz, *Advances in Echo-Contrast [1994(3)]*, pp 48-49).

WO-A-9320802 proposes that tissue-specific ultrasonic image enhancement may be achieved using

acoustically reflective oligolamellar liposomes conjugated to tissue-specific ligands such as antibodies, peptides, lectins etc. The liposomes are deliberately chosen to be devoid of gas and so will not have the advantageous echogenic properties of gas-based ultrasound contrast agents. Further references to this technology, e.g. in targeting to fibrin, thrombi and atherosclerotic areas are found in publications by Alkanonyuksel, H. *et al.* in *J. Pharm. Sci.* (1996) **85(5)**, 486-490; *J. Am. Coll. Cardiol.* (1996) **27(2) Suppl A**, 298A; and *Circulation, 68 Sci. Sessions*, Anaheim 13-16 November 1995.

There is also a number of publications concerning ultrasound contrast agents which refer in passing to possible use of monoclonal antibodies as vectors without giving significant practical detail and/or to reporters comprising materials which may be taken up by the reticuloendothelial system and thereby permit image enhancement of organs such as the liver - see, for example WO-A-9300933, WO-A-9401140, WO-A-9408627, WO-A-9428874, US-A-5088499, US-A-5348016 and US-A-5469854.

The present invention is based on the finding that gas-containing and gas-generating diagnostic and/or therapeutic agents coupled to non-bioactive vectors are particularly useful targeting agents by virtue of their enhanced safety relative to conventional targeting agents, which may elicit undesirable and unwanted biological effects in the subject.

An added advantage of certain kinds of non-bioactive vectors is that they may exhibit improved targeting efficacy as compared to bioactive vectors. The reason for this is that bioactive vectors usually will have to compete with endogenous ligands for the same binding site on the target molecule. In contrast non-bioactive vectors will often bind to target molecules for which endogenous ligands do not exist, or alternatively non-bioactive vectors may bind at sites in

a target molecule that is not involved in the biological function of the target molecule and for which natural ligands are not present in the body.

The term "non-bioactive" as used herein denotes two kinds of substances. First, it denotes a substance capable of interacting with or binding to a target molecule or target structure that is not normally involved in generating biological responses. Second, it denotes a substance capable of interacting with or binding to a given target without either giving or inhibiting the biological response normally elicited upon binding of a bioactive substance to the target, a bioactive substance being one which interacts with another molecule (i.e. a target) and generates a defined and measurable biological response.

Many receptors for transport proteins, such as receptors for transferrin or lipoproteins, do not in themselves generate a biological response upon binding of the ligand. Likewise a moiety carried in the protein may be bioactive, but the apoprotein itself is not. In a similar manner, many cofactors, vitamins etc. are bioactive only after they have been carried into the cell, that is to say, they do not elicit a biological response simply upon binding to the carrier protein.

Vectors useful in accordance with the invention may, for example, be non-bioactive *per se* or may be non-bioactive at doses useful for diagnostic and/or therapeutic purposes. Alternatively, a combination of otherwise bioactive vectors may be employed in such a way that a biological activity elicited by one type of vector is exactly counterbalanced by another kind or other kinds of vector molecules coupled to the same agent or otherwise present in the same diagnostic and/or therapeutic composition.

One advantageous embodiment of the invention is based on the additional finding that limited adhesion to targets is a highly useful property of diagnostic and/or

therapeutically active agents, which property may be achieved using non-bioactive vectors giving temporary retention rather than fixed adhesion to a target. Thus such agents, rather than being fixedly retained at specific sites, may for example effectively exhibit a form of retarded flow along the vascular endothelium by virtue of their transient interactions with endothelial cells. Such agents may thus become concentrated on the walls of blood vessels, in the case of ultrasound contrast agents providing enhanced echogenicity thereof relative to the bulk of the bloodstream, which is devoid of permanent structural features. They therefore may permit enhanced imaging of the capillary system, including the microvasculature, and so may facilitate distinction between normal and inadequately perfused tissue, e.g. in the heart, and may also be useful in visualising structures such as Kupffer cells, thrombi and atherosclerotic lesions or for visualising neo-vascularized and inflamed tissue areas. The present invention is also well suited to image changes occurring to normal blood vessels which are situated in areas of tissue necrosis.

Thus according to one aspect of the present invention there is provided a targetable diagnostic and/or therapeutic agent, e.g. an ultrasound contrast agent, comprising a suspension in an aqueous carrier liquid, e.g. an injectable carrier liquid, of a reporter comprising gas-containing or gas-generating material and coupled to one or more vectors, characterised in that said vector or vectors are non-bioactive.

Vectors useful in accordance with the invention include proteins which bind to cell-surface proteoglycans, e.g. so that the contrast agent becomes concentrated on cell surfaces. Such proteoglycans are large glycoproteins containing glucosaminoglycan side chains which, in many cases, are heparan sulphate.

Binding to glucosoaminoglycans has not been shown to elicit a biological response. In accordance with the invention one may isolate or synthesise the proteoglycan-binding (or, where appropriate, heparan sulphate-binding) part of the molecule for use as a vector, while avoiding any biological activity associated with other parts of the molecule.

The use of non-bioactive monomeric or oligomeric vectors and the use of non-bioactive peptide vectors represent preferred aspects of this invention.

A further aspect of the present invention is for example where a vector or vectors is attached to the reporter or included non-covalently into the reporter in a manner where the said vector or vectors is not readily exposed to the targets or receptors. Increased tissue specificity may therefore be achieved by applying an additional process to expose the vectors, e.g. the agent is exposed to external ultrasound to change the diffusibility of the moieties containing the vectors.

Any biocompatible gas may be present in the reporter of contrast agents according to the invention, the term "gas" as used herein including any substances (including mixtures) substantially or completely in gaseous (including vapour) form at the normal human body temperature of 37°C. The gas may thus, for example, comprise air; nitrogen; oxygen; carbon dioxide; hydrogen; an inert gas such as helium, argon, xenon or krypton; a sulphur fluoride such as sulphur hexafluoride, disulphur decafluoride or trifluoromethylsulphur pentafluoride; selenium hexafluoride; an optionally halogenated silane such as methylsilane or dimethylsilane; a low molecular weight hydrocarbon (e.g. containing up to 7 carbon atoms), for example an alkane such as methane, ethane, a propane, a butane or a pentane, a cycloalkane such as cyclopropane, cyclobutane or cyclopentane, an alkene such as ethylene, propene, propadiene or a butene, or an alkyne such as

acetylene or propyne; an ether such as dimethyl ether; a ketone; an ester; a halogenated low molecular weight hydrocarbon (e.g. containing up to 7 carbon atoms); or a mixture of any of the foregoing. Advantageously at least some of the halogen atoms in halogenated gases are fluorine atoms; thus biocompatible halogenated hydrocarbon gases may, for example, be selected from bromochlorodifluoromethane, chlorodifluoromethane, dichlorodifluoromethane, bromotrifluoromethane, chlorotrifluoromethane, chloropentafluoroethane, dichlorotetrafluoroethane, chlorotrifluoroethylene, fluoroethylene, ethylfluoride, 1,1-difluoroethane and perfluorocarbons, e.g. perfluoroalkanes such as perfluoromethane, perfluoroethane, perfluoropropanes, perfluorobutanes (e.g. perfluoro-n-butane, optionally in admixture with other isomers such as perfluoro-iso-butane), perfluoropentanes, perfluorohexanes and perfluoroheptanes; perfluoroalkenes such as perfluoropropene, perfluorobutenes (e.g. perfluorobut-2-ene) and perfluorobutadiene; perfluoroalkynes such as perfluorobut-2-yne; and perfluorocycloalkanes such as perfluorocyclobutane, perfluoromethylcyclobutane, perfluorodimethylcyclobutanes, perfluorotrimethyl-cyclobutanes, perfluorocyclopentane, perfluoromethyl-cyclopentane, perfluorodimethylcyclopentanes, perfluorocyclohexane, perfluoromethylcyclohexane and perfluorocycloheptane. Other halogenated gases include methyl chloride, fluorinated (e.g. perfluorinated) ketones such as perfluoroacetone and fluorinated (e.g. perfluorinated) ethers such as perfluorodiethyl ether. The use of perfluorinated gases, for example sulphur hexafluoride and perfluorocarbons such as perfluoropropane, perfluorobutanes and perfluoropentanes, may be particularly advantageous in view of the recognised high stability in the bloodstream of microbubbles containing such gases.

The reporter may be in any convenient form, for

example being any appropriate gas-containing or gas-generating ultrasound contrast agent formulation. Representative examples of such formulations include microbubbles of gas stabilised (e.g. at least partially encapsulated) by a coalescence-resistant surface membrane (for example gelatin, e.g. as described in WO-A-8002365), a filmogenic protein (for example an albumin such as human serum albumin, e.g. as described in US-A-4718433, US-A-4774958, US-A-4844882, EP-A-0359246, WO-A-9112823, WO-A-9205806, WO-A-9217213, WO-A-9406477 or WO-A-9501187), a polymer material (for example a synthetic biodegradable polymer as described in EP-A-0398935, an elastic interfacial synthetic polymer membrane as described in EP-A-0458745, a microparticulate biodegradable polyaldehyde as described in EP-A-0441468, a microparticulate N-dicarboxylic acid derivative of a polyamino acid - polycyclic imide as described in EP-A-0458079, or a biodegradable polymer as described in WO-A-9317718 or WO-A-9607434), a non-polymeric and non-polymerisable wall-forming material (for example as described in WO-A-9521631), or a surfactant (for example a polyoxyethylene-polyoxypropylene block copolymer surfactant such as a Pluronic, a polymer surfactant as described in WO-A-9506518, or a film-forming surfactant such as a phospholipid, e.g. as described in WO-A-9211873, WO-A-9217212, WO-A-9222247, WO-A-9428780 or WO-A-9503835).

Other useful gas-containing contrast agent formulations include gas-containing solid systems, for example microparticles (especially aggregates of microparticles) having gas contained therewithin or otherwise associated therewith (for example being adsorbed on the surface thereof and/or contained within voids, cavities or pores therein, e.g. as described in EP-A-0122624, EP-A-0123235, EP-A-0365467, WO-A-9221382, WO-A-9300930, WO-A-9313802, WO-A-9313808 or WO-A-

9313809). It will be appreciated that the echogenicity of such microparticulate contrast agents may derive directly from the contained/associated gas and/or from gas (e.g. microbubbles) liberated from the solid material (e.g. upon dissolution of the microparticulate structure).

The disclosures of all of the above-described documents relating to gas-containing contrast agent formulations are incorporated herein by reference.

Gas microbubbles and other gas-containing materials such as microparticles preferably have an initial average size not exceeding 10 μm (e.g. of 7 μm or less) in order to permit their free passage through the pulmonary system following administration, e.g. by intravenous injection.

Where phospholipid-containing compositions are employed in accordance with the invention, e.g. in the form of phospholipid-stabilised gas microbubbles, representative examples of useful phospholipids include lecithins (i.e. phosphatidylcholines), for example natural lecithins such as egg yolk lecithin or soya bean lecithin and synthetic or semisynthetic lecithins such as dimyristoylphosphatidylcholine, dipalmitoylphosphatidylcholine or distearoylphosphatidylcholine; phosphatidic acids; phosphatidylethanolamines; phosphatidylserines; phosphatidylglycerols; phosphatidylinositols; cardiolipins; sphingomyelins; fluorinated analogues of any of the foregoing; mixtures of any of the foregoing and mixtures with other lipids such as cholesterol. The use of phospholipids predominantly (e.g. at least 75%) comprising molecules individually bearing net overall charge, e.g. negative charge, for example as in naturally occurring (e.g. soya bean or egg yolk derived), semisynthetic (e.g. partially or fully hydrogenated) and synthetic phosphatidylserines, phosphatidylglycerols, phosphatidylinositols,

phosphatidic acids and/or cardiolipins, may be particularly advantageous.

Other exemplary lipids which may be used to prepare gas-containing contrast agents include fatty acids, stearic acid, palmitic acid, 2-n-hexadecylstearic acid, oleic acid and other acid containing lipid structures. These lipid structures are considered particularly interesting when coupled by amide bond formation to amino acids containing one or more amino groups. The resulting lipid modified amino acids (e.g. dipalmitoyllysine, distearoyl-2,3-diaminopropionic acid) are considered useful precursors for the attachment of functionalised spacer elements featuring coupling sites for conjugation of one or more vector molecules.

A further extension of this invention relates to the synthesis of lipopeptide structures comprising a lipid reporter attached to a linker portion (e.g. PEG, polyamino acid, alkylhalide etc) the said linker being suitably functionalised for coupling to one or more vector molecules. A particular preference is the inclusion of a positively charged linker element (eg. two or more lysine residues) for anchoring of the reporter element in the microbubble through electrostatic interaction with the negatively charged membrane.

Also considered within the scope of this invention are functionalised microbubbles carrying one or more reactive groups for non-specific modification of a multiplicity of receptor molecules located on cell surfaces. Microbubbles comprising a thiol moiety,for example, can bind to cell surface receptors via disulphide exchange reactions. The reversible nature of this covalent bond means that bubble flow can be controlled by altering the redox environment. Similarly `activated' microbubbles of membranes comprising active esters such as N-hydroxysuccinimide esters can be used to modify amino groups found on a multiplicity of cell

surface molecules.

Representative examples of gas-containing microparticulate materials which may be useful in accordance with the invention include carbohydrates (for example hexoses such as glucose, fructose or galactose; disaccharides such as sucrose, lactose or maltose; pentoses such as arabinose, xylose or ribose; α-, β- and γ-cyclodextrins; polysaccharides such as starch, hydroxyethyl starch, amylose, amylopectin, glycogen, inulin, pulullan, dextran, carboxymethyl dextran, dextran phosphate, ketodextran, aminoethyldextran, alginates, chitin, chitosan, hyaluronic acid or heparin; and sugar alcohols, including alditols such as mannitol or sorbitol), inorganic salts (e.g. sodium chloride), organic salts (e.g. sodium citrate, sodium acetate or sodium tartrate), X-ray contrast agents (e.g. any of the commercially available carboxylic acid and non-ionic amide contrast agents typically containing at least one 2,4,6-triiodophenyl group having substituents such as carboxyl, carbamoyl, N-alkylcarbamoyl, N-hydroxyalkylcarbamoyl, acylamino, N-alkylacylamino or acylaminomethyl at the 3- and/or 5-positions, as in metrizoic acid, diatrizoic acid, iothalamic acid, ioxaglic acid, iohexol, iopentol, iopamidol, iodixanol, iopromide, metrizamide, iodipamide, meglumine iodipamide, meglumine acetrizoate and meglumine diatrizoate), and polypeptides and proteins (e.g. gelatin or albumin such as human serum albumin).

The reporter may be made by any convenient process, for example by making gas-containing or gas-generating formulations. Representative examples include the preparation of a suspension of gas microbubbles by contacting a surfactant with gas and mixing them in the presence of an aqueous carrier, as described in WO 9115244; or by atomising a solution or dispersion of a wall-forming material in the presence of a gas in order

to obtain hollow microcapsules, as described in EP 512693A1; preparation of solid microspheres by á double emulsion process, as described in US 5648095; or a process for forming hollow microcapsules by spray-drying as described in EP 681843A2; or preparing gas-filled liposomes by shaking an aqueous solution comprising a lipid in the presence of a gas as described in US 5469854.

A suitable process for attachment of the desired vector to the reporter comprises a surface modification of the preformed reporter with a suitable linker employing reactive groups on the surface of both the reporter and vector. It may be particularly advantageous physically to mix the reporter material with the vector-containing substance at any step of the process. Such a process will result in incorporation or an attachment of the vector to the reporter. An optional process step may remove the excess of vector not bound to the reporter by washing the gas-containing particles following separation, by for example, floatation. A preferred aspect is the use of lipopeptide structures incorporating functional groups such as thiol, maleimide biotin etc. which can be premixed if desired with other reporter molecules before formation of gas-containing agents. The attachment of vector molecules may be carried out using the linker reagents listed below.

Coupling of a reporter unit to the desired vectors may be achieved by covalent or non-covalent means, usually involving interaction with one or more functional groups located on the reporter and/or vectors. Examples of chemically reactive functional groups which may be employed for this purpose include amino, hydroxyl, sulfhydryl, carboxyl, and carbonyl groups, as well as carbohydrate groups, vicinal diols, thioethers, 2-aminoalcohols, 2-aminothiols, guanidinyl, imidazolyl and phenolic groups.

Covalent coupling of reporter and vectors may

therefore be effected using linking agents containing reactive moities capable of reaction with such functional groups. Examples of reactive moieties capable of reaction with sulfhydryl groups include α-haloacetyl compounds of the type X-CH_2CO- (where X=Br, Cl or I), which show particular reactivity for sulfhydryl groups but which can also be used to modify imidazolyl, thioether, phenol and amino groups as described by Gurd, F.R.N. in *Methods Enzymol.* (1967) **11**, 532. N-Maleimide derivatives are also considered selective towards sulfhydryl groups, but may additionaly be useful in coupling to amino groups under certain conditions. N-maleimides may be incorporated into linking systems for reporter-vector conjugation as described by Kitagawa, T. *et al.* in *Chem. Pharm. Bull.* (1981) **29**, 1130 or used as polymer crosslinkers for bubble stabilisation as described by Kovacic, P. *et al.* in *J. Am. Chem. Soc.* (1959) **81**, 1887. Reagents such as 2-iminothiolane, e.g. as described by Traut, R. *et al.* in *Biochemistry* (1973) **12**, 3266, which introduce a thiol group through conversion of an amino group, may be considered as sulfhydryl reagents if linking occurs through the formation of disulphide bridges. Thus reagents which introduce reactive disulphide bonds into either the reporter or the vector may be useful, since linking may be brought about by disulphide exchange between the vector and reporter; examples of such reagents include Ellman's reagent (DTNB), 4,4'-dithiodipyridine, methyl-3-nitro-2-pyridyl disulphide and methyl-2-pyridyl disulphide (described by Kimura, T. *et al.* in *Analyt. Biochem.* (1982) **122**, 271).

Examples of reactive moieties capable of reaction with amino groups include alkylating and acylating agents. Representative alkylating agents include:

i) α-haloacetyl compounds, which show specificity towards amino groups in the absence of reactive thiol groups and are of the type X-CH_2CO- (where X=Cl, Br or

I), e.g. as described by Wong, Y-H.H. in *Biochemistry* (1979) **24**, 5337;

ii) N-maleimide derivatives, which may react with amino groups either through a Michael type reaction or through acylation by addition to the ring carbonyl group as described by Smyth, D.G. *et al.* in *J. Am. Chem.* Soc. (1960) **82**, 4600 and *Biochem. J.* (1964) **91**, 589;

iii) aryl halides such as reactive nitrohaloaromatic compounds;

iv) alkyl halides as described by McKenzie, J.A. *et al.* in *J. Protein Chem.* (1988) **7**, 581;

v) aldehydes and ketones capable of Schiff's base formation with amino groups, the adducts formed usually being stabilised through reduction to give a stable amine;

vi) epoxide derivatives such as epichlorohydrin and bisoxiranes,which may react with amino, sulfhydryl or phenolic hydroxyl groups;

vii) chlorine-containing derivatives of s-triazines, which are very reactive towards nucleophiles such as amino, sufhydryl and hydroxy groups;

viii) aziridines based on s-triazine compounds detailed above, e.g. as described by Ross, W.C.J. in *Adv. Cancer Res.* (1954) **2**, 1, which react with nucleophiles such as amino groups by ring opening;

ix) squaric acid diethyl esters as described by Tietze, L.F. in *Chem. Ber.* (1991) **124**, 1215; and

x) α-haloalkyl ethers, which are more reactive alkylating agents than normal alkyl halides because of the activation caused by the ether oxygen atom, e.g. as described by Benneche, T. *et al.* in Eur. J. Med. Chem. (1993) **28**, 463.

Representative amino-reactive acylating agents include:

i) isocyanates and isothiocyanates, particularly aromatic derivatives, Which form stable urea and thiourea derivatives respectively and have been used for

protein crosslinking as described by Schick, A.F. *et al.* in *J. Biol. Chem.* (1961) **236**, 2477;

ii) sulfonyl chlorides, which have been described by Herzig, D.J. *et al.* in *Biopolymers* (1964) **2**, 349 and which may be useful for the introduction of a fluorescent reporter group into the linker;

iii) Acid halides;

iv) Active esters such as nitrophenylesters or N-hydroxysuccinimidyl esters;

v) acid anhydrides such as mixed, symmetrical or N-carboxyanhydrides;

vi) other useful reagents for amide bond formation as described by Bodansky, M. *et al.* in *`Principles of Peptide Synthesis'* (1984) Springer-Verlag;

vii) acylazides, e.g. wherein the azide group is generated from a preformed hydrazide derivative using sodium nitrite, e.g. as described by Wetz, K. *et al.* in *Anal. Biochem.* (1974) **58**, 347;

viii) azlactones attached to polymers such as bis-acrylamide, e.g. as described by Rasmussen, J.K. in *Reactive Polymers* (1991) **16**, 199; and

ix) Imidoesters , which form stable amidines on reaction with amino groups, e.g. as described by Hunter, M.J. and Ludwig, M.L. in *J. Am. Chem. Soc.* (1962) **84**, 3491.

Carbonyl groups such as aldehyde functions may be reacted with weak protein bases at a pH such that nucleophilic protein side-chain functions are protonated. Weak bases include 1,2-aminothiols such as those found in N-terminal cysteine residues, which selectively form stable 5-membered thiazolidine rings with aldehyde groups, e.g. as described by Ratner, S. *et al.* in *J. Am. Chem. Soc.* (1937) **59**, 200. Other weak bases such as phenyl hydrazones may be used, e.g. as described by Heitzman, H. *et al.* in *Proc. Natl. Acad. Sci. USA* (1974) **71**, 3537.

Aldehydes and ketones may also be reacted with

amines to form Schiff's bases, which may advantageously be stabilised through reductive amination. Alkoxylamino moieties readily react with ketones and aldehydes to produce stable alkoxamines, e.g. as described by Webb, R. *et al.* in *Bioconjugate Chem.* (1990) **1**, 96.

Examples of reactive moieties capable of reaction with carboxyl groups include diazo compounds such as diazoacetate esters and diazoacetamides, which react with high specificity to generate ester groups, e.g. as described by Herriot R.M. in *Adv. Protein Chem.* (1947) **3**, 169. Carboxylic acid modifying reagents such as carbodiimides, which react through O-acylurea formation followed by amide bond formation, may also usefully be employed; linking may be facilitated through addition of an amine or may result in direct vector-receptor coupling. Useful water soluble carbodiimides include 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide (CMC) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), e.g. as described by Zot, H.G. and Puett, D. in *J. Biol. Chem.* (1989) **264**, 15552. Other useful carboxylic acid modifying reagents include isoxazolium derivatives such as Woodwards reagent K; chloroformates such as p-nitrophenylchloroformate; carbonyldiimidazoles such as 1,1'-carbonyldiimidazole; and N-carbalkoxydihydroquinolines such as N-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline.

Other potentially useful reactive moieties include vicinal diones such as p-phenylenediglyoxal, which may be used to react with guanidinyl groups, e.g. as described by Wagner *et al.* in *Nucleic acid Res.* (1978) **5**, 4065; and diazonium salts, which may undergo electrophilic substitution reactions, e.g. as described by Ishizaka, K. and Ishizaka T. in *J. Immunol.* (1960) **85**, 163. Bis-diazonium compounds are readily prepared by treatment of aryl diamines with sodium nitrite in acidic solutions. It will be appreciated that functional

groups in the reporter and/or vector may if desired be converted to other functional groups prior to reaction, e.g. to confer additional reactivity or selectivity. Examples of methods useful for this purpose include conversion of amines to carboxylic acids using reagents such as dicarboxylic anhydrides; conversion of amines to thiols using reagents such as N-acetylhomocysteine thiolactone, S-acetylmercaptosuccinic anhydride, 2-iminothiolane or thiol-containing succinimidyl derivatives; conversion of thiols to carboxylic acids using reagents such as α-haloacetates; conversion of thiols to amines using reagents such as ethylenimine or 2-bromoethylamine; conversion of carboxylic acids to amines using reagents such as carbodiimides followed by diamines; and conversion of alcohols to thiols using reagents such as tosyl chloride followed by transesterification with thioacetate and hydrolysis to the thiol with sodium acetate.

Vector-reporter coupling may also be effected using enzymes as zero-length crosslinking agents; thus, for example, transglutaminase, peroxidase and xanthine oxidase have been used to produce crosslinked products. Reverse proteolysis may also be used for crosslinking through amide bond formation.

Non-covalent vector-reporter coupling may, for example, be effected by electrostatic charge interactions e.g. between a polylysinyl-functionalised reporter and a polyglutamyl-functionalised vector, through chelation in the form of stable metal complexes or through high affinity binding interaction such as avidin/biotin binding. Polylysine, coated non-covalently to the negatively charged membrane surface can also increase non-specifically the affinity of a microbubble for a cell through charge interactions.

Alternatively, a vector may be coupled to a protein known to bind phospholipids. In many instances, a single molecule of phospholipid may attach to a

protein such as a translocase, while other proteins may attach to surfaces consisting mainly of phospholipid head groups and so may be used to attach vectors to phospholipid microspheres; one example of such a protein is ß2-glycoprotein I (Chonn, A., Semple, S.C. and Cullis, P.R., *Journal of Biological Chemistry* (1995) **270**, 25845-25849). Phosphatidylserine-binding proteins have been described, e.g. by Igarashi, K. *et al.* in *Journal of Biological Chemistry* **270(49)**, 29075-29078. Annexins are a class of phospholipid-binding proteins, many of which bind particularly avidly to phosphatidylserine (reviewed in Raynal, P. and H.B. Pollard. Annexins: the problem of assessing the biological role for a gene family of multifunctional calcium- and phospholipid-binding proteins". Biochim. Biophys. Acta **1197**: 63-93). A conjugate of a vector with such a phosphatidylserine-binding protein may therefore be used to attach the vector to phosphatidylserine-encapsulated microbubbles. When the amino acid sequence of a binding protein is known, the phospholipid-binding portion may be synthesised or isolated and used for conjugation with a vector, thus avoiding the biological activity which may be located elsewhere in the molecule.

It is also possible to obtain molecules that bind specifically to the surface (or in the "membrane") of microspheres by direct screening of molecular libraries for microsphere-binding molecules. For example, phage libraries displaying small peptides could be used for such selection. The selection may be made by simply mixing the microspheres and the phage display library and eluting the phages binding to the floating microspheres. If desired, the selection can be done under "physiological conditions" (e.g. in blood) to eliminate peptides which cross-react with blood components. An advantage of this type of selection procedure is that only binding molecules that do not destabilize the microspheres should be selected, since

only binding molecules attached to intact floating microspheres will rise to the top. It may also be possible to introduce some kind of "stress" during the selection procedure (e.g. pressure) to ensure that destabilizing binding moieties are not selected. Furthermore the selection could be done under shear conditions e.g. by first letting the phages react with the microspheres and then letting the microspheres pass through a surface coated with anti-phage antibodies under flow conditions. In this way it may be possible to select binders which may resist shear conditions present *in vivo*. Binding moieties identified in this way may be coupled (by chemical conjugation or via peptide synthesis, or at the DNA-level for recombinant vectors) to a vector molecule, constituting a general tool for attaching any vector molecule to the microspheres.

A vector which comprises or is coupled to a peptide, lipo-oligosaccharide or lipopeptide linker which contains a element capable of mediating membrane insertion may also be useful. One example is described by Leenhouts, J.M. *et al.* in *Febs Letters* (1995) **370(3)**, 189-192. Non-bioactive molecules consisting of known membrane insertion anchor/signal groups may also be used as vectors for certain applications, an example being the H1 hydrophobic segment from the Na,K-ATPase α-subunit described by Xie, Y. and Morimoto, T. in *J. Biol. Chem.* (1995) **270(20)**, 11985-11991. The anchor group may also be fatty acid(s) or cholesterol.

Coupling may also be effected using avidin or streptavidin, which have four high affinity binding sites for biotin. Avidin may therefore be used to conjugate vector to reporter if both vector and reporter are biotinylated. Examples are described by Bayer, E.A. and Wilchek, M. in *Methods Biochem. Anal.* (1980) **26**, 1.

This method may also be extended to include linking of reporter to reporter, a process which may

encourage bubble association and consequent potentially increased echogenicity. Alternatively, avidin or streptavidin may be attached directly to the surface of reporter microparticles.

Non-covalent coupling may also utilise the bifunctional nature of bispecific immunoglobulins. These molecules can specifically bind two antigens, thus linking them. For example, either bispecific IgG or chemically engineered bispecific F(ab)'2 fragments may be used as linking agents. Heterobifunctional bispecific antibodies have also been reported for linking two different antigens, e.g. as described by Bode, C. *et al.* in *J. Biol. Chem.* (1989) **264**, 944 and by Staerz, U.D. *et al.* in *Proc. Natl. Acad. Sci. USA* (1986) **83**, 1453. Similarly, any reporter and/or vector containing two or more antigenic determinants (e.g. as described by Chen, Aa *et al.* in *Am. J. Pathol.* (1988) **130**, 216) may be crosslinked by antibody molecules and lead to formation of multi-bubble cross-linked assemblies of potentially increased echogenicity.

Linking agents used in accordance with the invention will in general bring about linking of vector to reporter or reporter to reporter with some degree of specificity, and may also be used to attach one or more therapeutically active agents.

In some instances it is considered advantageous to include a PEG component as a stabiliser in conjunction with a vector or vectors or directly to the reporter in the same molecule where the PEG does not serve as a spacer.

Within the context of the present invention, the reporter unit will usually remain attached to the vectors. In another type of targeting procedure, sometimes called "pre-targeting", the vector (often, a monoclonal antibody) is administered alone; subsequently, the reporter is administered, coupled to a moiety which is capable of specifically binding the

vector molecule (when the vector is an antibody, the reporter may be coupled to an immunoglobulin-binding molecule, such as protein A or an anti-immunoglobulin antibody). The advantage of this protocol is that time may be allowed for elimination of the vector molecules that do not bind their targets, substantially reducing the background problems that are connected with the presence of an excess of reporter-vector conjugate. Within the context of the present invention, pre-targeting with one specific vector might be envisaged, followed by reporter units that are coupled to another vector *and* a moiety which binds the first vector.

Within the context of the present invention, in some cases and in particular for the assessment of blood perfusion rates in defined areas, for example in myocardium, it is of interest to measure the rate at which ultrasound contrast agents bound to the target are displaced or released from the target. This can be achieved in a controlled fashion by administration of the vector alone or other agents able to displace or release the ultarsound contrast agent from the target.

Ultrasound imaging modalities which may be used in accordance with the invention include two- and three-dimensional imaging techniques such as B-mode imaging (for example using the time-varying amplitude of the signal envelope generated from the fundamental frequency of the emitted ultrasound pulse, from sub-harmonics or higher harmonics thereof or from sum or difference frequencies derived from the emitted pulse and such harmonics, images generated from the fundamental frequency or the second harmonic thereof being preferred), colour Doppler imaging and Doppler amplitude imaging, and combinations of the two latter with any of the modalities (techniques) above. Surprisingly, the second harmonic signals from targeted monolayer microspheres were found to be excellent when used in accordance with the present invention. To reduce the

effects of movement, successive images of tissues such as the heart or kidney may be collected with the aid of suitable synchronisation techniques (e.g. gating to the ECG or respiratory movement of the subject). Measurement of changes in resonance frequency or frequency absorption which accompany arrested or retarded microbubbles may also usefully be made to detect the contrast agent

The present invention accordingly provides a tool for therapeutic drug delivery in combination with vector-mediated direction of the product to the desired site. By "therapeutic" or "drug" is meant an agent having a beneficial effect on a specific disease in a living human or non-human animal. Whilst combinations of drugs and ultrasound contrast agents have been proposed in, for example, WO-A-9428873 and WO-A-9507072, these products lack vectors having affinity for particular sites and thereby show comparitively poor specific retention at desired sites prior to or during drug release.

Therapeutic compounds used in accordance with the present invention may be encapsulated in the interior of the microbubbles/microparticles or attached to or incorporated into the structure thereof. Thus, the therapeutic compound may be linked to a part of the wall or matrix, for example through covalent or ionic bonds, or may be physically mixed into the encapsulating or matrix material, particularly if the drug has similar polarity or solubility to this material, so as to prevent it from leaking out of the product before it is intended to act in the body. The release of the drug may be initiated merely by wetting contact with blood following administration or as a consequence of other internal or external influences, e.g. dissolution processes catalyzed by enzymes or the use of ultrasound. The destruction of gas-containing microparticles using external ultrasound is a well known phenomenon in

respect of ultrasound contrast agents, e.g. as described in WO-A-9325241; the rate of drug release may be varied depending on the type of therapeutic application, using a specific amount of ultrasound energy from the transducer.

The therapeutic may be covalently linked to the membrane or matrix surface using a suitable linking agent, e.g. as described herein. Thus, for example, one may initially prepare a phospholipid or lipopeptide or derivative thereof to which the drug is bonded through a biodegradable bond or linker, and then incorporate this derivative into the material used to prepare the reporter, as described above. Alternatively, the product may initially be prepared without the therapeutic, which may then be coupled to or coated on the microbubbles or microparticles prior to use. Thus, for example, a therapeutic could be added to a suspension of microbubbles or microparticles in aqueous media and shaken in order to attach or adhere the therapeutic thereto.

Exemplary drug delivery systems suitable for use in the present compositions include any known therapeutic drugs or active analogues thereof containing thiol groups which are coupled to thiol containing microbubbles under oxidative conditions yielding disulphide bridges. In combination with a vector or vectors the drug/vector modified microbubbles are allowed to accumulate in the target tissue. Administration of a reducing agent such as reduced glutathione then liberates the drug molecule from the targeted microbubble in the vicinity of the target cell increasing the local concentration of the drug and enhancing therapeutic effect. The product may also be prepared without the therapeutic if desired. The drug may then be coupled to or coated on the microbubbles prior to use. Thus, for example, a therapeutic could be added to a suspension of microbubbles in aqueous media

and shaken in order to attach or adhere the therapeutic to the microbubbles.

Other drug delivery systems include vector modified phospholipid membranes doped with lipopeptide structures comprising a poly-L-lysine or poly-D-lysine chain in combination with a targeting vector. Applied to gene therapy/antisense technologies with particular emphasis on receptor-mediated drug delivery the microbubble carrier is condensed with DNA or RNA via elecrostatic interaction with the polycation. This method has the advantage that the vector or vectors used for targeted delivery are not directly attached to the polysine carrier moiety. The polylysine chain is also anchored more tightly in the microbubble membrane due to the presence of the lipid chains. The use of ultrasound to increase the effectiveness of delivery is also considered useful.

Alternatively free polylysine chains are firstly modified with drug or vector molecules then condensed onto the negative surface of targeted microbubbles.

Representative and non-limiting examples of drugs useful in accordance with the invention include antineoplastic agents such as vincristine, vinblastine, vindesine, busulfan, chlorambucil, spiroplatin, cisplatin, carboplatin, methotrexate, adriamycin, mitomycin, bleomycin, cytosine arabinoside, arabinosyl adenine, mercaptopurine, mitotane, procarbazine, dactinomycin (antinomycin D), daunorubicin, doxorubicin hydrochloride, taxol, plicamycin, aminoglutethimide, estramustine, flutamide, leuprolide, megestrol acetate, tamoxifen, testolactone, trilostane, amsacrine (m-AMSA), asparaginase (L-asparaginase), etoposide, interferon a-2a and 2b, blood products such as hematoporphyrins or derivatives of the foregoing; biological response modifiers such as muramylpeptides; antifungal agents such as ketoconazole, nystatin, griseofulvin, flucytosine, miconazole or amphotericin B; hormones or

hormone analogues such as growth hormone, melanocyte stimulating hormone, estradiol, beclomethasone dipropionate, betamethasone, cortisone acetate, dexamethasone, flunisolide, hydrocortisone, methylprednisolone, paramethasone acetate, prednisolone, prednisone, triamcinolone or fludrocortisone acetate; vitamins such as cyanocobalamin or retinoids; enzymes such as alkaline phosphatase or manganese superoxide dismutase; antiallergic agents such as amelexanox; inhibitors of tissue factor such as monoclonal antibodies and Fab fragments thereof, synthetic peptides, nonpeptides and compounds downregulating tissue factor expression; inhibitors of platelets such as, GPIa, GPIb and GPIIb-IIIa, ADP receptors, thrombin receptors, von Willebrand factor, prostaglandins, aspirin, ticlopidin, clopigogrel and reopro; inhibitors of coagulation protein targets such as: FIIa FVa, FVIIa, FVIIIA, FIXa, tissue factor, hepatins, hirudin, hirulog, argatroban, DEGR-rFVIIa and annexin V; inhibitors of fibrin formation and promoters of fibrionolysis such as t-PA, urokinase, Plamin, Streptokinase, rt-Plasminogen Activator and rStaphylokinase; antiangiogenic factors such as medroxyprogesteron, pentosan polysulphate, suramin, taxol, thalidomide, angiostatin, interferon-alpha, metalloproteinase inhibitors, platelet factor 4, somatostatin, thromobospondin; circulatory drugs such as propranolol; metabolic potentiators such as glutathione; antituberculars such as p-aminosalicylic acid, isoniazid, capreomycin sulfate, cyclosexine, ethambutol, ethionamide, pyrazinamide, rifampin or streptomycin sulphate; antivirals such as acyclovir, amantadine, azidothymidine, ribavirin or vidarabine; blood vessel dilating agents such as diltiazem, nifedipine, verapamil, erythritol tetranitrate, isosorbide dinitrate, nitroglycerin or pentaerythritol tetranitrate; antibiotics such as dapsone, chloramphenicol, neomycin, cefaclor, cefadroxil,

cephalexin, cephradine, erythromycin, clindamycin, lincomycin, amoxicillin, ampicillin, bacampicillin, carbenicillin, dicloxacillin, cyclacillin, picloxacillin, hetacillin, methicillin, nafcillin, penicillin, polymyxin or tetracycline; antiinflammatories such as diflunisal, ibuprofen, indomethacin, meclefenamate, mefenamic acid, naproxen, phenylbutazone, piroxicam, tolmetin, aspirin or salicylates; antiprotozoans such as chloroquine, metronidazole, quinine or meglumine antimonate; antirheumatics such as penicillamine; narcotics such as paregoric; opiates such as codeine, morphine or opium; cardiac glycosides such as deslaneside, digitoxin, digoxin, digitalin or digitalis; neuromuscular blockers such as atracurium mesylate, gallamine triethiodide, hexafluorenium bromide, metocurine iodide, pancuronium bromide, succinylcholine chloride, tubocurarine chloride or vecuronium bromide; sedatives such as amobarbital, amobarbital sodium, apropbarbital, butabarbital sodium, chloral hydrate, ethchlorvynol, ethinamate, flurazepam hydrochloride, glutethimide, methotrimeprazine hydrochloride, methyprylon, midazolam hydrochloride, paraldehyde, pentobarbital, secobarbital sodium, talbutal, temazepam or triazolam; local anaesthetics such as bupivacaine, chloroprocaine, etidocaine, lidocaine, mepivacaine, procaine or tetracaine; general anaesthetics such as droperidol, etomidate, fentanyl citrate with droperidol, ketamine hydrochloride, methohexital sodium or thiopental and pharmaceutically acceptable salts (e.g. acid addition salts such as the hydrochloride or hydrobromide or base salts such as sodium, calcium or magnesium salts) or derivatives (e.g. acetates) thereof. Other examples of therapeutics include genetic material such as nucleic acids, RNA, and DNA of natural or synthetic origin, including recombinant RNA and DNA. DNA encoding certain proteins may be used in the treatment of many different types of

diseases. For example, tumor necrosis factor or interleukin-2 genes may be provided to treat advanced cancers; thymidine kinase genes may be provided to treat ovarian cancer or brain tumors; interleukin-2 genes may be provided to treat neuroblastoma, malignant melanoma or kidney cancer; and interleukin-4 genes may be provided to treat cancer.

Lipophilic derivatives of drugs linked to the microbubble wall through hydrophobic interactions may exhibit therapeutic effects as part of the microbubble or after release from the microbubble, e.g. by use of ultrasound. If the drug does not possess the desired physical properties, a lipophilic group may be introduced for anchoring the drug to the membrane. Preferably the lipophilic group should be introduced in a way that does not influence the *in vivo* potency of the molecule, or the lipophilic group may be cleaved releasing the active drug. Lipophilic groups may be introduced by various chemical means depending on functional groups available in the drug molecule. Covalent coupling may be effected using functional groups in the drug molecule capable of reacting with appropriately functionalised lipophilic compounds. Examples of lipophilic moieties include branched and unbranched alkyl chains, cyclic compounds, aromatic residues and fused aromatic and non-aromatic cyclic systems. In some instances the lipophilic moiety will consist of a suitably functionalised steroid, like cholesterol and related compounds. Examples of functional groups particularly suitable for derivatisation include nucleophilic groups like amino, hydroxy and sulfhydryl groups. Suitable processes for lipophilic derivatisation of any drug containing a sulfhydryl group, like captopril, may include direct alkylation, e.g. reaction with an alkyl halide under basic conditions and thiol ester formation by reaction with an activated carboxylic acid. Representative

examples of derivatisation of any drug having carboxylic functions, like atenolol and chlorambucil, include amide and ester formation by coupling of amines and alcohols, respectively, possesing requested physical properties. A preferred aspect is attachment of cholesterol to a therapeutic compound by forming a degradable ester bond.

A preferred application of the present invention relates to *angiogenesis*, which is the formation of new blood vessels by branching from existing vessels. The primary stimulus for this process may be inadequate supply of nutrients and oxygen (hypoxia) to cells in a tissue. The cells may respond by secreting angiogenetic factors, of which there are many; one example is *vascular endothelial growth factor*. These factors initiate the secretion of proteolytic enzymes which break down the proteins of the basement membrane, as well as inhibitors which limit the action of these potentially harmful enzymes. The combined effect of loss of attachment and signals from the receptors for angiogenetic factors is to cause the endothelial cells to move, multiply, and rearrange themselves, and finally to synthetise a basement membrane around the new vessels.

Tumors must initiate angiogenesis when they reach millimeter size in order to keep up their rate of growth. As angiogenesis is accompanied by characteristic changes in the endothelial cells and their environment, this process is a promising target for therapeutic intervention. The transformations accompanying angiogenesis are also very promising for diagnosis, a preferred example being malignant disease, but the concept also shows great promise in inllammation and a variety of inflammation-related diseases. These factors are also involved in re-vascularisation of infarcted parts of the myocardium. which occurs if the stenosis is released within a short time.

A number of known receptors/targets associated

with angiogenesis are given in subsequent tables. Using the targeting principles described in the present disclosure, angiogenesis mav be detected by the majority of the imaging modalities in use in medicine. Contrast-enhanced ultrasound may possess additional advantages, the contrast medium being microspheres which are restricted to the interior of blood vessels. Even if the target antigens are found on many cell types, the microspheres will attach exclusively to endothelial cells.

So-called prodrugs may also be used in agents according to the invention. Thus drugs may be derivatised to alter their physicochemical properties and to adapt them for inclusion into the reporter; such derivatised drugs may be regarded as prodrugs and are usually inactive until cleavage of the derivatising group regenerates the active form of the drug.

By targeting a gas-filled microbubble containing a prodrug-activating enzyme to areas of pathology one may image targeting of the enzyme, making it possible to visualise when the micobubbles are targeted properly to the area of pathology and at the same time have disappeared from non-target areas. In this way one can determine the optimal time for injection of prodrug into individual patients.

Another alternative is to incorporate the prodrug, the prodrug-activating enzyme and the vector in the same microbubble in a system where the prodrug will only be activated after some external stimulus. Such a stimulus may, for example, be a tumour-specific protease as described above, or bursting of the bubbles by external ultrasound after the desired targeting has been achieved.

Therapeutics may easily be delivered in accordance with the invention to diseased and necrotic areas including the heart and vasculature in general, and to

the liver, spleen and kidneys and other regions such as the lymph system, body cavities or gastrointestinal system.

Products according to the present invention may be used for targeted therapeutic delivery either *in vivo* or *in vitro*. In the latter context the products may be useful in *in vitro* systems such as kits for diagnosis of different diseases or characterisation of different components in blood or tissue samples. Similar techniques to those used to attach certain blood components or cells to polymer particles(e.g. monodisperse magnetic particles) *in vitro* to separate them from a sample may be used in the present invention,using the low density of the reporter units in agents of the present invention to effect separation of the gas-containing material by floatation and repeated washing.

So-called zero-length linking agents, which induce direct covalent joining of two reactive chemical groups without introducing additional linking material (e.g. as in amide bond formation induced using carbodiimides or enzymatically) may, if desired, be used in accordance with the invention, as may agents such as biotin/avidin systems which induce non-covalent reporter-vector linking and agents which induce hydrophobic or electrostatic interactions.

Most commonly, however, the linking agent will comprise two or more reactive moieties, e.g. as described above, connected by a spacer element. The presence of such a spacer permits bifunctional linkers to react with specific functional groups within a molecule or between two different molecules, resulting in a bond between these two components and introducing extrinsic linker-derived material into the reporter-vector conjugate. The reactive moieties in a linking agent may be the same (homobifunctional agents) or different (heterobifunctional agents or, where several

dissimilar reactive moieties are present, heteromultifunctional agents), providing a diversity of potential reagents that may bring about covalent bonding between any chemical species, either intramolecularly or intermolecularly.

The nature of extrinsic material introduced by the linking agent may have a critical bearing on the targeting ability and general stability of the ultimate product. Thus it may be desirable to introduce labile linkages, e.g. containing spacer arms which are biodegradable or chemically sensitive or which incorporate enzymatic cleavage sites. Alternatively the spacer may include polymeric components, e.g. to act as surfactants and enhance bubble stability. The spacer may also contain reactive moieties, e.g. as described above to enhance surface crosslinking, or it may contain a tracer element such as a fluorescent probe, spin label or radioactive material.

Contrast agents according to the present invention are therefore useful in all imaging modalities since contrast elements such as X-ray contrast agents, light imaging probes, spin labels or radioactive units may readily be incorporated in or attached to the reporter units.

Spacer elements may typically consist of aliphatic chains which effectively separate the reactive moieties of the linker by distances of between 5 and 30 Å. They may also comprise macromolecular structures such as poly(ethylene glycols). Such polymeric structures, hereinafter referred to as PEGs, are simple, neutral polyethers which have been given much attention in biotechnical and biomedical applications (see e.g. Milton Harris, J. (ed) "*Poly(ethylene glycol) chemistry, biotechnical and biomedical applications*" Plenum Press, New York, 1992). PEGs are soluble in most solvents, including water, and are highly hydrated in aqueous environments, with two or three water molecules bound to

each ethylene glycol segment; this has the effect of preventing adsorption either of other polymers or of proteins onto PEG-modified surfaces. PEGs are known to be nontoxic and not to harm active proteins or cells, whilst covalently linked PEGs are known to be non-immunogenic and non-antigenic. Furthermore, PEGs may readily be modified and bound to other molecules with only little effect on their chemistry. Their advantageous solubility and biological properties are apparent from the many possible uses of PEGs and copolymers thereof, including block copolymers such as PEG-polyurethanes and PEG-polypropylenes.

Appropriate molecular weights for PEG spacers used in accordance with the invention may, for example, be between 120 Daltons and 20 kDaltons.

The major mechanism for uptake of particles by the cells of the reticuloendothelial system (RES) is opsonisation by plasma proteins in blood; these mark foreign particles which are then taken up by the RES. The biological properties of PEG spacer elements used in accordance with the invention may serve to increase contrast agent circulation time in a similar manner to that observed for PEGylated liposomes (see e.g. Klibanov, A.L. *et al.* in *FEBS Letters* (1990) **268**, 235-237 and Blume, G. and Cevc, G. in *Biochim. Biophys. Acta* (1990) **1029**, 91-97). Increased coupling efficiency to areas of interest may also be achieved using antibodies bound to the terminii of PEG spacers (see e.g. Maruyama, K. *et al.* in *Biochim. Biophys. Acta* (1995) **1234**, 74-80 and Hansen, C.B. *et al.* in *Biochim. Biophys. Acta* (1995) **1239**, 133-144).

In some instances it is considered advantageous to include a PEG component as a stabiliser in conjunction with a vector or vectors or directly to the reporter in the same molecule where the PEG does not serve as a spacer.

Other representative spacer elements include

structural-type polysaccharides such as polygalacturonic acid, glycosaminoglycans, heparinoids, cellulose and marine polysaccharides such as alginates, chitosans and carrageenans; storage-type polysaccharides such as starch, glycogen, dextran and aminodextrans; polyamino acids and methyl and ethyl esters thereof, as in homo- and co-polymers of lysine, glutamic acid and aspartic acid; and polypeptides, oligonucleotides and oligosaccharides, which may or may not contain enzyme cleavage sites.

In general, spacer elements may contain cleavable groups such as vicinal glycol, azo, sulfone, ester, thioester or disulphide groups. Spacers containing biodegradable methylene diester or diamide groups of formula

$$-(Z)_m.Y.X.C(R^1R^2).X.Y.(Z)_n-$$

[where X and Z are selected from -O-, -S-, and -NR- (where R is hydrogen or an organic group); each Y is a carbonyl, thiocarbonyl, sulphonyl, phosphoryl or similar acid-forming group: m and n are each zero or 1; and R^1 and R^2 are each hydrogen, an organic group or a group $-X.Y.(Z)_m-$, or together form a divalent organic group] may also be useful; as discussed in, for example, WO-A-9217436 such groups are readily biodegraded in the presence of esterases, e.g. *in vivo*, but are stable in the absence of such enzymes. They may therefore advantageously be linked to therapeutic agents to permit slow release thereof.

Poly[N-(2-hydroxyethyl)methacrylamides] are potentially useful spacer materials by virtue of their low degree of interaction with cells and tissues (see e.g. Volfová, I., Říhová, B. and V.R. and Vetvicka, P. in *J. Bioact. Comp. Polymers* (1992) **7**, 175-190). Work on a similar polymer consisting mainly of the closely related 2-hydroxypropyl derivative showed that it was endocytosed by the mononuclear phagocyte system only to a rather low extent (see Goddard, P., Williamson, I.,

Bron, J., Hutchkinson, L.E., Nicholls, J. and Petrak, K. in *J. Bioct. Compat. Polym.* (1991) **6**, 4-24.).

Other potentially useful poymeric spacer materials include:

i) copolymers of methyl methacrylate with methacrylic acid; these may be erodible (see Lee, P.I. in *Pharm. Res.* (1993) **10**, 980) and the carboxylate substituents may cause a higher degree of swelling than with neutral polymers;

ii) block copolymers of polymethacrylates with biodegradable polyesters (see e.g. San Roman, J. and Guillen-Garcia, P. in *Biomaterials* (1991) **12**, 236-241);

iii) cyanoacrylates, i.e. polymers of esters of 2-cyanoacrylic acid - these are biodegradable and have been used in the form of nanoparticles for selective drug delivery (see Forestier, F., Gerrier, P., Chaumard, C., Quero, A.M., Couvreur, P. and Labarre, C. in *J. Antimicrob. Chemoter.* (1992) **30**, 173-179);

iv) polyvinyl alcohols, which are water-soluble and generally regarded as biocompatible (see e.g. Langer, R. in *J. Control. Release* (1991) **16**, 53-60);

v) copolymers of vinyl methyl ether with maleic anhydride, which have been stated to be bioerodible (see Finne, U., Hannus, M. and Urtti, A. in *Int. J. Pharm. (1992)* **78**. 237-241);

vi) polyvinylpyrrolidones, e.g. with molecular weight less than about 25,000, which are rapidly filtered by the kidneys (see Hespe, W., Meier, A. M. and Blankwater, Y. M. in *Arzeim.-Forsch./Drug Res. (1977)* **27**, 1158-1162);

vii) polymers and copolymers of short-chain aliphatic hydroxyacids such as glycolic, lactic, butyric, valeric and caproic acids (see e.g. Carli, F. in *Chim. Ind. (Milan)* (1993) **75**, 494-9), including copolymers which incorporate aromatic hydroxyacids in order to increase their degradation rate (see Imasaki, K., Yoshida, M., Fukuzaki, H., Asano, M., Kumakura, M., Mashimo, T.,

Yamanaka, H. and Nagai. T. in *Int. J. Pharm.* (1992) **81**, 31-38);

viii) polyesters consisting of alternating units of ethylene glycol and terephthalic acid, e.g. Dacron^{R}, which are non-degradable but highly biocompatible;

ix) block copolymers comprising biodegradable segments of aliphatic hydroxyacid polymers (see e.g. Younes, H., Nataf, P.R., Cohn, D., Appelbaum, Y.J., Pizov, G. and Uretzky, G. in *Biomater. Artif. Cells Artif. Organs* (1988) **16**, 705-719), for instance in conjunction with polyurethanes (see Kobayashi, H., Hyon, S.H. and Ikada, Y. in "Water-curable and biodegradable prepolymers" - *J. Biomed. Mater. Res.* (1991) **25**, 1481-1494);

x) polyurethanes, which are known to be well-tolerated in implants, and which may be combined with flexible "soft" segments, e.g. comprising poly(tetra methylene glycol), poly(propylene glycol) or poly(ethylene glycol)) and aromatic "hard" segments, e.g. comprising 4,4'-methylenebis(phenylene isocyanate) (see e.g. Ratner, B.D., Johnston, A.B. and Lenk, T.J. in *J. Biomed. Mater. Res: Applied Biomaterials* (1987) **21**, 59-90; Sa Da Costa, V. *et al.* in *J. Coll. Interface Sci.* (1981) **80**, 445-452 and Affrossman, S. *et al.* in *Clinical Materials* (1991) **8**, 25-31);

xi) poly(1,4-dioxan-2-ones), which may be regarded as biodegradable esters in view of their hydrolysable ester linkages (see e.g. Song, C. X., Cui, X. M. and Schindler, A. in *Med. Biol. Eng. Comput.* (1993) **31**, S147-150), and which may include glycolide units to improve their absorbability (see Bezwada, R.S., Shalaby, S.W. and Newman, H.D.J. in *Agricultural and synthetic polymers: Biodegradability and utilization* **(1990)** (ed Glass, J.E. and Swift, G.), 167-174 - ACS symposium Series, #433, Washington D.C., U.S.A. - American Chemical Society);

xii) polyanhydrides such as copolymers of sebacic acid (octanedioic acid) with bis(4-carboxy-phenoxy)propane,

which have been shown in rabbit studies (see Brem, H., Kader, A., Epstein, J.I., Tamargo, R.J., Domb, A., Langer, R. and Leong, K.W. in *Sel. Cancer Ther.* (1989) **5**, 55-65) and rat studies (see Tamargo, R.J., Epstein, J.I., Reinhard, C.S., Chasin, M. and Brem, H. in *J. Biomed. Mater. Res.* (1989) **23**, 253-266) to be useful for controlled release of drugs in the brain without evident toxic effects;

xiii) biodegradable polymers containing ortho-ester groups, which have been employed for controlled release *in vivo* (see Maa, Y.F. and Heller, J. in *J. Control. Release* (1990) **14**, 21-28); and

xiv) polyphosphazenes, which are inorganic polymers consisting of alternate phosphorus and nitrogen atoms (see Crommen, J.H., Vandorpe, J. and Schacht, E.H. in *J. Control. Release* (1993) **24**, 167-180).

The following tables list linking agents, biotinylation agents and agents for protein modification which may be useful in preparing targetable agents in accordance with the invention.

Heterobifunctional linking agents

Linking agent	Reactivity 1	Reactivity 2	Comments
ABH	carbohydrate	photoreactive	
ANB-NOS	$-NH_2$	photoreactive	
APDP(1)	-SH	photoreactive	iodinable disulphide linker
APG	$-NH_2$	photoreactive	reacts selectively with Arg at pH 7-8
ASIB(1)	-SH	photoreactive	iodinable
ASBA(1)	-COOH	photoreactive	iodinable

EDC	$-NH_2$	-COOH	zero-length linker
GMBS	$-NH_2$	-SH	
sulfo-GMBS	$-NH_2$	-SH	water-soluble
HSAB	$-NH_2$	photoreactive	
sulfo-HSAB	$-NH_2$	photoreactive	water-soluble
MBS	$-NH_2$	-SH	
sulfo-MBS	$-NH_2$	-SH	water-soluble
M_2C_2H	carbohydrate	-SH	
MPBH	carbohydrate	-SH	
NHS-ASA(1)	$-NH_2$	photoreactive	iodinable
sulfo-NHS-ASA(1)	$-NH_2$	photoreactive	water-soluble, iodinable
sulfo-NHS-LC-ASA(1)	$-NH_2$	photoreactive	water-soluble, iodinable
PDPH	carbohydrate	-SH	disulphide linker
PNP-DTP	$-NH_2$	photoreactive	
SADP	$-NH_2$	photoreactive	disulphide linker
sulfo-SADP	$-NH_2$	photoreactive	water-soluble disulphide linker
SAED	$-NH_2$	photoreactive	disulphide linker
SAND	$-NH_2$	photoreactive	water-soluble disulphide linker
SANPAH	$-NH_2$	photoreactive	
sulfo-SANPAH	$-NH_2$	photoreactive	water-soluble

SASD(1)	$-NH_2$	photoreactive	water-soluble iodinable disulphide linker
SIAB	$-NH_2$	-SH	
sulfo-SIAB	$-NH_2$	-SH	water-soluble
SMCC	$-NH_2$	-SH	
sulfo-SMCC	$-NH_2$	-SH	water-soluble
SMPB	$-NH_2$	-SH	
sulfo-SMPB	$-NH_2$	-SH	water-soluble
SMPT	$-NH_2$	-SH	
sulfo-LC-SMPT	$-NH_2$	-SH	water-soluble
SPDP	$-NH_2$	-SH	
sulfo-SPDP	$-NH_2$	-SH	water-soluble
sulfo-LC-SPDP	$-NH_2$	-SH	water-soluble
sulfo-SAMCA(2)	$-NH_2$	photoreactive	
sulfo-SAPB	$-NH_2$	photoreactive	water-soluble

Notes: (1)=iodinable; (2)=fluorescent

Homobifunctional linking agents

Linking agent	Reactivity	Comments
BS	$-NH_2$	
BMH	-SH	
BASED(i)	photoreactive	iodinable disulphide linker
BSCOES	$-NH_2$	
sulfo-BSCOES	$-NH_2$	water-soluble
DFDNB	$-NH_2$	
DMA	$-NH_2$	
DMP	$-NH_2$	
DMS	$-NH_2$	

DPDPB	-SH	disulphide linker
DSG	$-NH_2$	
DSP	$-NH_2$	disulphide linker
DSS	$-NH_2$	
DST	$-NH_2$	
sulfo-DST	$-NH_2$	water-soluble
DTBP	$-NH_2$	disulphide linker
DTSSP	$-NH_2$	disulphide linker
EGS	$-NH_2$	
sulfo-EGS	$-NH_2$	water-soluble
SPBP	$-NH_2$	

Biotinylation agents

Agent	Reactivity	Comments
biotin-BMCC	-SH	
biotin-DPPE*		preparation of biotinylated liposomes
biotin-LC-DPPE*		preparation of biotinylated liposomes
biotin-HPDP	-SH	disulphide linker
biotin-hydrazide	carbohydrate	
biotin-LC-hydrazide	carbohydrate	
iodoacetyl-LC-biotin	$-NH_2$	
NHS-iminobiotin	$-NH_2$	reduced affinity for avidin
NHS-SS-biotin	$-NH_2$	disulphide linker
photoactivatable biotin	nucleic acids	
sulfo-NHS-biotin	$-NH_2$	water-soluble
sulfo-NHS-LC-biotin	$-NH_2$	

Notes: DPPE=dipalmitoylphosphatidylethanolamine; LC=long chain

Agents for protein modification

Agent	Reactivity	Function
Ellman's reagent	-SH	quantifies/detects/protects
DTT	-S.S-	reduction
2-mercaptoethanol	-S.S-	reduction
2-mercaptylamine	-S.S-	reduction
Traut's reagent	$-NH_2$	introduces -SH
SATA	$-NH_2$	introduces protected -SH
AMCA-NHS	$-NH_2$	fluorescent labelling
AMCA-hydrazide	carbohydrate	fluorescent labelling
AMCA-HPDP	-S.S-	fluorescent labelling
SBF-chloride	-S.S-	fluorescent detection of -SH
N-ethylmaleimide	-S.S-	blocks -SH
NHS-acetate	$-NH_2$	blocks and acetylates $-NH_2$
citraconic anhydride	$-NH_2$	reversibly blocks and introduces negative charges
DTPA	$-NH_2$	introduces chelator
BNPS-skatole	tryptophan	cleaves tryptophan residue
Bolton-Hunter	$-NH_2$	introduces iodinable group

Other potentially useful protein modifications include partial or complete deglycosidation by neuraminidase, endoglycosydases or periodate, since deglycosidation often results in less uptake by liver, spleen, macrophages etc., whereas neo-glycosylation of proteins often results in increased uptake by the liver and macrophages); preparation of truncated forms by proteolytic cleavage, leading to reduced size and shorter half life in circulation; and cationisation, e.g. as described by Kumagi *et al.* in *J. Biol. Chem.* (1987) **262**, 15214-15219; Triguero *et al.* in *Proc. Natl. Acad. Sci. USA* (1989) **86**, 4761-4765; Pardridge *et al.* in *J. Pharmacol. Exp. Therap.* (1989) **251**, 821-826 and

Pardridge and Boado, *Febs Lett.* (1991) **288**, 30-32.

Vectors which may be usefully employed in targetable agents according to the invention include the following:

i) Antibodies, which can be used as vectors for a very wide range of targets, and which have advantageous properties such as very high specificity, high affinity (if desired), the possiblity of modifying affinity according to need etc. Whether or not antibodies will be bioactive will depend on the specific vector/target combination. Both conventional and genetically engineered antibodies may be employed, the latter permitting engineering of antibodies to particular needs, e.g. as regards affinity and specificity. The use of human antibodies may be preferred to avoid possible immune reactions against the vector molecule. A further useful class of antibodies comprises so-called bi- and multi-specific antibodies, i.e. antibodies having specificity for two Or more different antigens in one antibody molecule. Such antibodies may, for example, be useful in promoting formation of bubble clusters and may also be used for various therapeutic purposes, e.g. for carrying toxic moieties to the target. Various aspects of bispecific antibodies are described by McGuinness, B.T. *et al.* in *Nat. Biotechnol.* (1996) **14**, 1149-1154; by George, A.J. *et al.* in *J. Immunol.* (1994) **152**, 1802-1811; by Bonardi *et al.* in *Cancer Res.* (1993) **53**, 3015-3021; and by French, R.R. *et al.* in *Cancer Res.* (1991) **51**, 2353-2361.

ii) Cell adhesion molecules, their receptors, cytokines, growth factors, peptide hormones and fragments and pieces thereof. Such vectors rely on normal biological protein-protein interactions with their target molecules, and so in many cases will generate a biological response on binding with the

targets and thus be bioactive; this may be a relatively insignificant concern with vectors which target proteoglycans.

iii) Non-bioactive binders of receptors for cell adhesion molecules, cytokines, growth factors or peptide hormones. This category may include peptidic or non-peptidic non-bioactive vectors which will be neither agonists nor antagonists.

iv) Oligonucleotides and modified oligonucleotides which bind DNA or RNA through Watson-Crick or other types of base-pairing. DNA is usually only present in extracelluar space as a consequence of cell damage, so that such oligonucleotides, which will usually be non-bioactive, may be useful in, for example, targeting of necrotic regions, which are associated with many different pathological conditions. Oligonucleotides may also be designed to bind to specific DNA- or RNA-binding proteins, for example transcription factors which are very often highly overexpressed or activated in tumour cells or in activated immune or endothelial cells. Combinatorial libraries may be used to select oligonucleotides which bind specifically to any possible target molecules (from the examples of proteins to caffeine) and which therefore may be employed as vectors for targeting.

v) DNA-binding drugs may behave similarly to oligonuclotides, but may exhibit biological acitvity and/or toxic effects if taken up by cells.

vi) Protease substrates/inhibitors. Proteases are involved in many pathological conditions. Many substrates/inhibitors are non-peptidic but, at least in the case of inhibitors, are often bioactive.

vii) Vector molecules may be generated from combinatorial libraries without necessarily knowing the exact molecular target, by functionally selecting (in vitro, ex vivo or in vivo) for molecules binding to the region/structure to be imaged.

viii) Various small molecules, including bioactive compounds known to bind to biological receptors of various kinds. Such vectors or their targets may be used for generate non-bioactive compounds binding to the same targets.

ix) proteins or peptides which bind to glucosaminoglycan side chains eg heparan sulphate, including glucosoaminoglycan-binding portions of larger molecules, as binding to glucosoaminoglycans does not result in a biological response. Proteoglycans are not found on red blood cells, which eliminates undesirable adsorption to these cells.

The following tables identify various vectors which may be targeted to particular types of targets and indicated areas of use for targetable diagnostic and/or therapeutic agents according to the invention which contain such vectors. In the cases where the vectors stated are bioactive it is understood that either: (1) non-bioactive analogs are used; (2) the vectors are used in doses too low to generate a biological response; or (3) the vectors are used in combinations in such a way that the resulting diagnostic and/or therapeutic composition gives no biological response.

Protein and peptide vectors - antibodies

Vector type	Target	Comments/areas of use	Ref

antibodies (general)	CD34	vascular diseases in general, normal vessel wall (e.g myocardium), activated endothelium, immune cells	1
"	ICAM-1	"	1
"	ICAM-2	"	1
"	ICAM-3	"	1
"	E-selectin	"	1
"	P-selectin	"	1
"	PECAM	"	1
"	Integrins, e.g. VLA-1, VLA-2, VLA-3, VLA-4, VLA-5, VLA-6, $\beta_1\alpha_7$, $\beta_1\alpha_8$, $\beta_1\alpha_v$, LFA-1, Mac-1, CD41a, etc.	"	2
"	GlyCAM	Vessel wall in lymph nodes (quite specific for lymph nodes)	3
"	MadCam 1	"	3
"	fibrin	Thrombi	4
"	Tissue Factor	Activated endothelium, tumours	5
"	Myosin	Necrosis, myocardial infaction	6
"	CEA (carcino-embryonal antigen)	Tumours	7
"	Mucins	Tumours	8
"	Multiple drug resistance protein	Tumours	9
"	Prostate specific antigen	Prostate cancer	

"	Cathepsin B	Tumours (proteases of various kinds are often more or less specifically overexpressed in a variety of tumours - Cathepsin B is such a protease)	10
"	Transferrin receptor	Tumors, vessel wall	11
MoAb 9.2.27		Tumours Antigen upregulated on cell growth	12
	VAP-1	Adhesion molecule	13
	Band 3 protein	Upregulated during phagocytic activity	
antibodies	CD44	tumour cells	a
antibodies	β2-micro-globulin	general	b
antibodies	MHC class 1	general	b
antibodies	integrin αvβ3	tumours, angiogenesis	c

a.) Heider, K. H., M. Sproll, S. Susani, E. Patzelt, P. Beaumier, E. Ostermann, H. Ahorn, and G. R. Adolf. **1996.** "Characterization of a high-affinity monoclonal antibody specific for CD44v6 as candidate for immunotherapy of squamous cell carcinomas". *Cancer Immunology Immunotherapy* **43**: 245-253.

b). I. Roitt, J. Brostoff, and D. Male. **1985.** *Immunology*, London: Gower Medical Publishing, p. 4.7

c.) Stromblad, S., and D. A. Cheresh. **1996.** "Integrins, angiogenesis and vascular cell survival". *Chemistry & Biology* **3**: 881-885.

Protein and peptide vectors - cell adhesion molecules etc.

Vector type	Target	Comments/areas of use	Ref

L-selectin	CD34 MadCAM1 GlyCam 1	vascular diseases in general, normal vessel wall (e.g myocardium), activated endothelium, Lymph nodes	3
Other selectins	carbohydrate ligands (sialyl Lewis x) heparan sulfate	vascular diseases in general, normal vessel wall (e.g myocardium), activated endothelium	14
RGD-peptides	integrins	"	2
PECAM	PECAM, and other	Endothelium, Cells in immune system	15
Integrins, e.g. VLA-1, VLA-2, VLA-3, VLA-4, VLA-5, VLA-6, $\beta_1\alpha_7$, $\beta_1\alpha_8$, $\beta_1\alpha_v$, LFA-1, Mac-1, CD41a, etc.	Laminin, collagen, fibronectin, VCAM-1, thrombospondin, vitronectin etc.	Endothelium, Vessel wall etc.	16
Integrin receptors, e.g.Laminin, collagen, fibronectin, VCAM-1, thrombospondin, vitronectin etc.	Integrins, e.g. VLA-1, VLA-2, VLA-3, VLA-4, VLA-5, VLA-6, $\beta_1\alpha_7$, $\beta_1\alpha_8$, $\beta_1\alpha_v$, LFA-1, Mac-1, CD41a, etc.	Cells in immune system vessel wall etc.	1718
Nerve cell adhesion molecule (N-CAM) RGD-peptides	proteoglycans N-CAM (homophilic) integrins	angiogenesis	19 c

<u>Vectors comprising cytokines/growth factors/peptide hormones and fragments thereof</u>

Vector type	Target	Comments/areas of use	Ref
Epidermal growth factor	EGF-receptor or related receptors	Tumours	20
Nerve growth factor	NGF-receptor	Tumours	21
Somatostatin	ST-receptor	Tumours	22
Endothelin	Endothelin-receptor	Vessel wall	
Interleukin-1	IL-1-receptor	Inflammation, activated cells of different kinds	23
Interleukin-2	IL-2-receptor	"	24
Chemokines (*ca.* 20 different cytokines partly sharing receptors)	Chemokine receptors, proteoglycans	Inflammation	25
Tumour necrosis factor	TNF-receptors	Inflammation	
Parathyroid hormone	PTH-receptors	Bone diseases Kidney diseases	
Bone Morphogenetic Protein	BMP-receptors	Bone Diseases	
Calcitonin	CT-receptors	Bone diseases	
Colony stimulating factors (G-CSF, GM-CSF, M-CSF, IL-3)	Corresponding specific receptors, proteoglycans	Endothelium	26
Insulin like growth factor I	IGF-I receptor	Tumours, other growing tissues	
Atrial Natriuretic Factor	ANF-receptors	Kidney, vessel wall	

Vasopressin	Vasopressin receptor	Kidney, vessel wall	
VEGF	VEGF-receptor	Endothelium, regions of angiogenesis	
Fibroblast growth factors	FGF-receptors, Proteoglycans	Endothelium Angiogenesis	27
Schwann cell growth factor	proteoglycans specific receptors		28

Miscellaneous protein and peptide vectors

Vector type	Target	Comments/areas of use	Ref
Streptavidin	Kidney	Kidney diseases	29
Bacterial fibronectin-binding proteins	Fibronectin	Vessel wall	30
Fc-part of antibodies	Fc-receptors	Monocytes macrophages liver	31
Transferrin	transferrin-receptor	Tumours vessel walls	11
Streptokinase/ tissue plasminogen activator	thrombi	thrombi	
Plasminogen, plasmin	Fibrin	Thrombi, tumours	32
Mast cell proteinases	proteoglycans		33
Elastase	proteoglycans		34
Lipoprotein lipase	proteoglycans		35
Coagulation enzymes	proteoglycans		36
Extracellular superoxide dismutase	proteoglycans		37
Heparin cofactor II	proteoglycans		38
Retinal survival factor	proteoglycans specific receptors		39

Heparin-binding brain mitogen	proteoglycans specific receptors		40
Apolipoprotein, e.g. apolipoprotein B	proteoglycans specific receptors (e.g., LDL receptor)		41
Apolipoprotein E	LDL receptor proteoglycans		42
Adhesion-promoting proteins, e.g. Purpurin	proteoglycans		43
Viral coat proteins, e.g. HIV, Herpes	proteoglycans		44
Microbial adhesins, e.g. "Antigen 85" complex of mycobacteria	fibronectin, collagen, fibrinogen, vitronectin, heparan sulfate		45
ß-amyloid precursor	proteoglycans	ß-amyloid accumulates in Alzheimer's disease	46
Tenascin, e.g .tenascin C	heparan sulfate, integrins		47

Vectors comprising non-peptide agonists/antagonists or non-bioactive binders of receptors for cytokines/growth factors/peptide hormones/cell adhesion molecules

Vector type	Target	Comments/areas of use	Ref
		Several agonists/antagonists are known for such factors acting through G-protein coupled receptors	48 49
Endothelin antagonist	Endothelin receptor	Vessel wall	
Desmopressin (vasopressin analogue)	Vasopressin receptor	Kidney Vessel wall	
Demoxytocin (oxytocin analogue)	Oxytocin Receptor	Reproductive organs, Mammary glands, Brain	
Angiotensin II receptor antagonists CV-11974, TCV-116	Angiotensin II receptors	Vessel wall brain adrenal gland	
non-peptide RGD-analogues	integrins	Cells in immune system vessel wall etc.	50

Vectors comprising anti-angiogenic factors

Vector type	Target	Comments/areas of use	Ref
Angiostatin	EC of tumors	plasminogen fragment	K
cartilage-derived inhibitor	EC of tumors		J

β-Cyclodextrin tetradecasulfate	tumors, inflammation		C
fumagillin and analogs	tumors, inflammation		E
Interferon-α	EC of tumors		K
Interferon-γ	EC of tumors		E
interleukin-12	EC of tumors		E
linomide	tumors, inflammation		A
medroxyprogesterone	EC of tumors		K
metalloproteinase inhibitors	EC of tumors		K
pentosan polysulfate	EC of tumors		K
platelet factor 4	EC of tumors		M
Somatostatin	EC of tumors		K
Suramin	EC of tumors		K
Taxol	EC of tumors		K
thalidomide	EC of tumors		K
Thrombospondin	EC of tumors		K

Vectors comprising angiogenic factors

Vector type	Target	Comments/areas of use	Ref
acidic fibroblast growth factor	EC of tumors		K
adenosine	EC of tumors		K
Angiogenin	EC of tumors		K
Angiotensin II	EC of tumors		K
basement membrane components	tumors	e.g., tenascin, collagen IV	M
basic fibroblast growth factor	EC of tumors		K

Bradykinin	EC of tumors		K
Calcitonin gene-related peptide	EC of tumors		K
epidermal growth factor	EC of tumors		K
Fibrin	tumors		K
Fibrinogen	tumors		K
Heparin	EC of tumors		K
histamine	EC of tumors		K
hyaluronic acid or fragments thereof	EC of tumors		K
Interleukin-1α	EC of tumors		K
laminin, laminin fragments	EC of tumors		K
nicotinamide	EC of tumors		K
platelet activating factor	EC of tumors		K
Platelet-derived endothelial growth factor	EC of tumors		K
prostaglandins E1, E2	EC of tumors		K
spermine	EC of tumors		K
spermine	EC of tumors		K
Substance P	EC of tumors		K
transforming growth factor-α	EC of tumors		K
transforming growth factor-β	EC of tumors		K
Tumor necrosis factor-α	EC of tumors		K
vascular endothelial growth factor/vascular permeability factor	EC of tumors		K
vitronectin			A

Vector molecules other than recognized angiogenetic factors with known affinity for receptors associated with angiogenesis

Vector type	Target	Comments/areas of use	Ref
angiopoietin	tumors, inflammation		B
α_2-antiplasmin	tumors, inflammation		
combinatorial libraries, compounds from	tumors, inflammation	for instance: compounds that bind to basement membrane after degradation	
endoglin	tumors, inflammation		D
endosialin	tumors, inflammation		D
endostatin [collagen fragment]	tumors, inflammation		M
Factor VII related antigen	tumors, inflammation		D
fibrinopeptides	tumors, inflammation		ZC
fibroblast growth factor, basic	tumors, inflammation		E
hepatocyte growth factor	tumors, inflammation		I
insulin-like growth factor	tumors, inflammation		R
interleukins	tumors, inflammation	e.g.,: IL-8	I
leukemia inhibitory factor	tumors, inflammation		A

metalloproteinase inhibitors	tumors, inflammation	e.g., batimastat	E
Monoclonal antibodies	tumors, inflammation	for instance: to angiogenetic factors or their receptors, or to components of the fibrinolytic system	
peptides, for instance cyclic RGDᴅFV	tumors, inflammation		B,Q
placental growth factor	tumors, inflammation		J
placental proliferin-related protein	tumors, inflammation		E
plasminogen	tumors, inflammation		M
plasminogen activators	tumors, inflammation		D
plasminogen activator inhibitors	tumors, inflammation		U,V
platelet activating factor antagonists	tumors, inflammation	inhibitors of angiogenesis	A
platelet-derived growth factor	tumors, inflammation		E
pleiotropin	tumors, inflammation		ZA
proliferin	tumors, inflammation		E
proliferin related protein	tumors, inflammation		E
selectins	tumors, inflammation	e.g., E-selectin	D
SPARC	tumors, inflammation		M

snake venoms (RGD-containing)	tumors, inflammation		Q
Tissue inhibitor of metalloproteinases	tumors, inflammation	e g,, TIMP-2	U
thrombin	tumors, inflammation		H
thrombin-receptor-activating tetradecapeptide	tumors, inflammation		H
thymidine phosphorylase	tumors, inflammation		D
tumor growth factor	tumors, inflammation		ZA

Receptors/targets associated with angiogenesis

Vector type	Target	Comments/areas of use	Ref
biglycan	tumors, inflammation	dermatan sulfate proteoglycan	X
CD34	tumors, inflammation		L
CD44	tumors, inflammation		F
collagen type I, IV, VI, VIII	tumors, inflammation		A
decorin	tumors, inflammation	dermatan sulfate proteoglycan	Y
dermatan sulfate proteoglycans	tumors, inflammation		X
endothelin	tumors, inflammation		G
endothelin receptors	tumors, inflammation		G

fibronectin	tumors		P
Flk-1/KDR, Flt-4	tumors, inflammation	VEGF receptor	D
FLT-1 (fms-like tyrosine kinase)	tumors, inflammation	VEGF-A receptor	O
heparan sulfate	tumors, inflammation		p
hepatocyte growth factor receptor (c-met)	tumors, inflammation		I
insulin-like growth factor/mannose-6-phosphate receptor	tumors, inflammation		R
integrins: β_3 and β_5, integrin $\alpha_v\beta_3$, integrin $\alpha_6\beta_1$, , integrins α_6, integrins β_1, integrin $\alpha_2\beta_1$, integrin $\alpha_v\beta_3$, integrin α_5 integrin $\alpha_v\beta_5$, fibrin receptors.	Tumors, inflammation	laminin receptor subunit of the fibronectin receptor	D, P
Intercellular adhesion molecule-1 and -2	tumors, inflammation		P
Jagged gene product	tumors, inflammation		T
Ly-6	tumors, inflammation	a lymphocyte activation protein	N
matrix metalloproteinases	tumors, inflammation		D
MHC class II	tumors, inflammation		

Notch gene product	tumors, inflammation		T
Osteopontin	tumors		Z
PECAM	tumors, inflammation	alias CD31	P
plasminogen activator receptor	tumors, inflammation		ZC
platelet-derived growth factor receptors	tumors, inflammation		E
Selectins: E-, P-	tumors, inflammation		D
Sialyl Lewis-X	tumors, inflammation	blood group antigen	M
stress proteins: glucose regulated, heat shock families and others	tumors, inflammation	molecular chaperones	
syndecan	tumors, inflammation		T
thrombospondin	tumors, inflammation		M
TIE receptors	tumors, inflammation	tyrosine kinases with Ig- and EGF-like domains	E
tissue factor	tumors, inflammation		Z
tissue inhibitor of metalloproteinases	tumors, inflammation	e.g., TIMP-2	U
transforming growth factor receptor	tumors, inflammation		E
urokinase-type plasminogen activator receptor	tumors, inflammation		D
Vascular cellular adhesion molecule (VCAM)	tumors, inflammation		D

Vascular endothelial growth factor related protein	tumors, inflammation		
Vascular endothelial growth factor-A receptor	tumors, inflammation		K
von Willebrand factor-related antigen	tumors, inflammation		L

Oligonucleotide vectors

Vector type	Target	Comments/areas of use	Ref
Oligonucleotides complementary to repeated sequences, e.g. genes for ribosomal RNA, Alu-sequences	DNA made available by necrosis	Tumours Myocardial infarction All other diseases that involves necrosis	51
Oligonucleotides complementary to disease-specific mutations (e.g. mutated oncogenes).	DNA made available by necrosis in a region of the relevant disease	Tumours	51
Oligonucleotides complementary to DNA of infecting agent.	DNA of infective agent	Viral or bacterial infections	51
Triple or quadruple-helix forming oligonucleotides	As in above examples	As in above examples	51

Oligonucleotides with recognition sequence for DNA-or RNA- binding proteins	DNA-binding protein, e.g. transcription factors (often overexpressed/ activated in tumours or activated endothelium/ immune cells	Tumours Activated endothelium Activated immune cells	

Modified oligonucleotide vectors

Vector type	Target	Comments/areas of use	Ref
Phosphorothioate oligos	As for unmodified oligos	As for unmodified oligos	51
2'-O-methyl substituted oligos	"	"	51
circular oligos	"	"	51
oligos containing hairpin structure to decrease degradation	"	"	51
oligos with terminal phosphorothioate	"	"	51
2'-fluoro oligos	"	"	51
2'-amino oligos	"	"	51
DNA-binding drugs conjugated to oligos (for examples, see below)	"	Increased binding affinity as compared to pure oligos	52

Peptide Nucleic Acids (PNAs, oligonucleotidss with a peptide backbone)	"	Increased binding affinity and stability compared to standard oligos.	53

Nucleoside and nucleotide vectors

Vector type	Target	Comments/areas of use	Ref
Adenosine or analogues	Adenosine receptors	Vessel wall Heart	54
ADP, UDP, UTP and others	Various nucleotide receptors	Many tissues, e.g. brain, spinal cord, kidney, spleen	55

Receptors comprising DNA-binding drugs

Vector type	Target	Comments/areas of use	Ref
acridine derivatives distamycin netropsin actinomycin D echinomycin bleomycin etc.	DNA made available by necrosis	Tumours, Myocardial infarction and all other diseases involving necrosis or other processes liberating DNA from cells	

Receptors comprising protease substrates

Vector type	Target	Comments/areas of use	Ref
Peptidic or non-peptidic substrates	Cathepsin B	Tumours, a variety of which may more or less specifically overexpress proteases of various kinds, e.g. Cathepsin B	10

Receptors comprising protease inhibitors

Vector type	Target	Comments/areas of use	Ref
Peptidic or non-peptidic inhibitors e.g. N-acetyl-Leu-Leu-norleucinal	Cathepsin B	Tumours, a variety of which may more or less specifically overexpress proteases of various kinds, e.g. Cathepsin B	10
bestatin ([(2S,3R)-3-Amino-2-hydroxy-4-phenyl-butanoyl]-L-leucine hydrochloride)	Aminopeptidases	Tumours, e.g. on cell surfaces	
Pefabloc (4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride)	Serine proteases	Tumours, vessel wall etc.	
Commercially available inhibitors e.g. kaptopril enalapril ricionopril	Angiotensin converting enzyme	Endothelial cells	

Low specificity non-peptidic compounds	Coagulation factors	Vessel wall injury, tumours, etc.	
Protease nexins (extracellular protease inhibitors)	proteoglycans		56
Antithrombin	proteoglycans, Coagulation factors		57

Vectors from combinatorial libraries

Vector type	Target	Comments/areas of use	Ref
Antibodies with structure determined during generation process	Any of above targets - or may be unknown when make functional selection of vector binding to chosen diseased structure	Any diseased or normal structure of interest, e.g. thrombi, tumours or walls of myocardial vessels	58, 59, 60
Peptides with sequence determined during generation process	"	"	58, 59, 60
Oligonucleotides with sequence determined during generation process	"	"	58, 59, 60
Modifications of oligos obtained as above	"	"	58, 59, 60

Other chemicals with structure determined during generation process	"	"	58, 59, 60

Carbohydrate vectors

Vector type	Target	Comments/areas of use	Ref
neo-glycoproteins	macrophages	general activation/ inflammation	
oligosaccharides with terminal galactose	Asialo-glycoprotein receptor	liver	61
Hyaluronan	aggrecan (a proteoglycan) "link proteins" cell-surface receptors: CD44		62
Mannose		Blood brain barrier, Brain tumours and other diseases causing changes in BBB	63
Bacterial glycopeptides		"	64

(Glyco)Lipid vectors

Vector type	Target	Comments/areas of use	Ref
GM1 gangliosides	cholera bacteria in the gastrointestinal tract	diagnosis/treatment of cholera	

platelet activating factor (PAF) antagonists	PAF receptors	diagnosis of inflammation	
Prostoglandin antagonists of inflammation	Prostoglandin receptors	diagnosis of inflammation	
Thromboxane antagonists of inflammation	Leukotriene receptors	diagnosis of inflammation	

Small molecule vectors

Vector type	Target	Comments/areas of use	Ref
Adrenalin	Corresponding receptors		
Betablockers	Adrenergic beta-receptors	Myocardium for beta-1 blockers	
Alpha-blockers	Adrenergic alpha-receptors	Vessel wall	
benzodiazepines			
serotonin-analogues	Serotonin-receptors		
anti-histamines	Histamine-receptors	Vessel wall	
Acetyl-choline receptor antagonists	ACh-receptors		
verapamil	Ca^{2+}-channel blocker	Heart muscle	
nifedipin	Ca^{2+}-channel blocker	Heart muscle	

Amiloride	Na^+/H^+-exchanger	Blocks this exchanges in kidney and is generally upregulated in cells stimulated by growth factors.	
Digitalis glycosides	Na^+/K^+-ATP-ases	myocardium peripheral vasculature, central nervous system	
Thromboxae/ Prostaglandin receptor antagonists or agonists	Thromboxane/ prostaglandin receptors	Vessel wall, Endothelium	
Glutathione	Glutathione-receptors Leukotriene-receptors	Lung, Brain	
Biotin	biotin transport protein on cell surface		65
Folate	folate transport protein on cell surface	Tumours	66
Riboflavin	riboflavin transport protein on cell surface		67

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The following non-limitative examples serve to illustrate the invention. Confirmation of the microparticulate nature of products is performed using microscopy as described in WO-A-9607434. Ultrasonic transmission measurements may be made using a broadband transducer to indicate suspensions of products giving an increased sound beam attenuation compared to a standard. Flow cytometric analysis of products can be used to confirm attachment of macromolecules thereto. The ability of targeted agents to bind specifically to cells expressing a target may be studied by microscopy and/or using a flow chamber containing immobilised cells, for example employing a population of cells expressing the target structure and a further population of cells not expressing the target. Radioactive or fluorescent enzyme-labelled streptavidin/avidin may used to analyse biotin attachment.

Example 1 - Gas-filled microbubbles encapsulated with phosphatidylserine, phosphatidylcholine and biotin-amidocaproate-PEG_{3400}-Ala-cholesterol

a) Synthesis of Z-Ala-cholesterol (3-O-(carbobenzyloxy-L-alanyl)cholesterol)

Cholesterol (4mmol), Z-alanine (5 mmol) and dimethylaminopyridine (4 mmol) were dissolved in dimethylformamide/tetrahydrofuran (20 ml + 5 ml) and dicyclohexylcarbodiimide was added. The reaction mixture was stirred at ambient temperature overnight. Dicyclohexylurea was filtered off and the solvent was rotary evaporated. The residue was taken up in chloroform, undissolved dicyclohexylurea was filtered off and the solvent was removed by rotary evaporation. The residue was placed on a column of silica gel, and Z-Ala-cholesterol was eluted with toluene/petroleum ether (20:2) followed by toluene/diethyl ether (20:2). The fractions containing the title compound were combined and the solvent was removed by rotary evaporation. The structure of the product was confirmed by NMR.

b) Synthesis of Ala-cholesterol (3-O-(L-alanyl)-cholesterol)

Z-Ala-cholesterol (0.48 mmol) is placed in tetrahydrofuran (20 ml) and glacial acetic acid (3 ml) and hydrogenated in the presence of 5 % palladium on charcoal for 2 hours. The reaction mixture is filtered and concentrated *in vacuo*.

c) Synthesis of Boc-NH-PEG_{3400}-Ala-cholesterol

Ala-cholesterol is added to a solution of Boc-NH-PEG_{3400}-SC (t-butyl carbamate poly(ethylene glycol)-succinimidyl carbonate) (Shearwater) in chloroform, followed by triethylamine. The suspension is stirred at

41 °C for 10 minutes. The crude product is purified by chromatography.

d) Synthesis of H_2N-PEG_{3400}-Ala-cholesterol

Boc-NH-PEG_{3400}-Ala-cholesterol is stirred in 4 M hydrochloric acid in dioxane for 2.5 hours at ambient temperature. The solvent is removed by rotary evaporation and the residue is taken up in chloroform and washed with water. The organic phase is rotary evaporated to dryness. The crude product may be purified by chromatography.

e) Synthesis of biotinamidocaproate-PEG_{3400}-Ala-cholesterol

A solution of biotinamidocaproate N-hydroxysuccinimide ester in tetrahydrofuran is added to H_2N-PEG_{3400}-Ala-cholesterol dissolved in tetrahydrofuran and 0.1 M sodium phosphate buffer having a pH of 7.5 (2 ml). The reaction mixture is heated to 30 °C and the reaction is followed to completion by TLC, whereafter the solvent is evaporated.

f) Preparation of gas-filled microbubbles encapsulated with phosphatidylserine, phosphatidylcholine and biotinamidocaproate-PEG_{3400}-Ala-cholesterol

To a mixture (5 mg) of phosphatidylserine and phosphatidylcholine (in total 90-99.9mol%) and biotinamidocaproate-PEG_{3400}-Ala-cholesterol (10-0.1mol%) is added 5% propyleneglycol-glycerol in water (1 ml). The dispersion is heated to not more than 80 °C for 5 minutes and then cooled to ambient temperature. The dispersion (0.8 ml) is then transferred to a vial (1 ml) and the head space is flushed with perfluorobutane. The vial is shaken in a cap-mixer for 45 seconds, whereafter

the sample is put on a roller table. After centrifugation the infranatant is exchanged with water and the washing is repeated.

g) Alternative preparation of gas-filled microbubbles encapsulated with phosphatidylserine, phosphatidylcholine and biotinamidocaproate-PEG_{3400}-Ala-cholesterol

To a mixture (5 mg) of phosphatidylserine and phosphatidylcholine is added 5% propyleneglycol-glycerol in water (1 ml). The dispersion is heated to not more than 80 °C for 5 minutes and then cooled to ambient temperature. The dispersion (0.8 ml) is then transferred to a vial (1 ml) and the head space is flushed with perfluorobutane. The vial is shaken in a cap-mixer for 45 seconds, whereafter the sample is put on a roller table. After centrifugation the infranatant is exchanged with water. Biotinamidocaproate-PEG_{3400}-Ala-cholesterol dissolved in water is added the washed microbubbles, which are placed on a roller table for several hours. The washing procedure is repeated following incorporation of the biotinamidocaproate-PEG_{3400}-Ala-cholesterol into the microbubble membranes.

Example 2 - Gas-containing microparticles comprising phosphatidylserine, phosphatidylcholine, biotin-amidocaproate-PEG_{3400}-Ala-Cholesterol and drug-cholesterol

a) Synthesis of drug-cholesterol

Cholesterol (4mmol), a drug having an acid group (see Example 2(b) for a list of cholesterol-derivatised drugs) and dimethylaminopyridine (4 mmol) are dissolved in dimethylformamide/tetrahydrofuran (20 ml + 5 ml) and dicyclohexylcarbodiimide is added. The reaction mixture is stirred at ambient temperature overnight.

Dicyclohexylurea is filtered off and the solvent is rotary evaporated. The title compound is purified by chromatography.

b) Preparation of gas-filled microbubbles encapsulated with phosphatidylserine, phosphatidylcholine, biotinamidocaproate-PEG_{3400}-Ala-cholesterol and drug-cholesterol

To a mixture (5 mg) of phosphatidylserine and phosphatidylcholine (in total 90-99.9mol%) and biotinamidocaproate-PEG_{3400}-Ala-cholesterol (prepared as in Example 1) and drug-cholesterol (in total 10-0.1mol%) is added 5% propyleneglycol-glycerol in water (1 ml). The dispersion is heated to not more than 80 °C for 5 minutes and then cooled to ambient temperature. The dispersion (0.8 ml) is transferred to a vial (1 ml) and the head space is flushed with perfluorobutane. The vial is shaken in a cap-mixer for 45 seconds whereafter the sample is put on a roller table. After centrifugation the infranatant is exchanged with water and the washing is repeated.

Example 3 - Biotin attached to gas-filled microbubbles

Biotin may be attached to microbubbles in many different ways, e.g. in a similar way to that described by Corley, P. and Loughrey, H.C. in (1994) *Biochim. Biophys. Acta* **1195**, 149-156. The resulting bubbles are analysed by flow cytometry, e.g. by employing fluorescent streptavidin to detect attachment of biotin to the bubbles. Alternatively radioactive or enzyme-labelled streptavidin/avidin is used to analyse biotin attachment.

Example 4 -Gas-filled microbubbles encapsulated with 1.2-distearoyl-sn-Glycero-3-[Phospo-L-Serine] and

biotin-DPPE

1.2-distearoyl-sn-Glycero-3-[Phospo-L-Serine] (Avanti lot# 180PS-12, 22.6 mg) was added 4% propylenglycol-glycerol in water (4 ml). The dispersion was heated, to not more than 80 °C for five minutes, and then cooled to ambient temperature. An aqueous dispersion of biotin-DPPE (Pierce lot# 96092472, 1.5 mg) in 4% propylenglycol-glycerol (1 ml) was added and the sample was put on a roller table for 1-2 hours. The suspension was filled on vials and head spaces were flushed with perfluorobutane. The vials were shaken for 45 seconds whereafter they were put on a roller table. After centrifugation for seven minutes the infranatant was exchanged with water and the washing was repeated two times.

Normal Phase HPLC with an Evaporative Light Scattering Detector confirmed that the membranes of the microbubbles contained 4 mol% biotin-DPPE. The mean particle diameter of the microbubbles was 4 μm measured by Coulter Counter. Ultrasound transmission measurements using a 3.5 MHz broadband transducer showed that a particle dispersion of < 2 mg/ml gave a sound beam attenuation higher than 5 dB/cm.

Example 5 - Gas-filled microbubbles encapsulated with phosphatidylserine and biotinylated oligonucleotide non-covalently bound to streptavidin-Succ-PEG-DSPE

a) Synthesis of Succ-PEG_{3400}-DSPE

NH_2-PEG_{3400}-DSPE (prepared as in Preparation 1) is carboxylated using succinic anhydride, e.g. by a similar method to that described by Nayar, R. and Schroit, A.J. in *Biochemistry* (1985) **24**, 5967-5971.

b) Preparation of gas-filled microbubbles encapsulated with phosphatidylserine and Succ-PEG_{3400}-DSPE

To a mixture (5 mg) of phosphatidylserine (90-99.9 mol%) and Succ-PEG_{3400}-DSPE (10-0.1 mol%) is added 5% propyleneglycol-glycerol in water (1 ml). The dispersion is heated to not more than 80 °C for 5 minutes and then cooled to ambient temperature. The dispersion (0.8 ml) is transferred to a vial (1 ml) and the head space is flushed with perfluorobutane. The vial is shaken in a cap-mixer for 45 seconds, whereafter the sample is put on a roller table. After centrifugation the infranatant is exchanged with water and the washing is repeated. Alternatively the microbubbles may be prepared as described in Preparation 1(f).

c) Coupling of streptavidin to gas-filled microbubbles encapsulated with phosphatidylserine and Succ-PEG_{3400}-DSPE

Streptavidin is covalently bound to succ-PEG_{3400}-DSPE in the microbubbles by standard coupling methods using a water-soluble carbodiimide. The sample is placed on a roller table during the reaction. After centrifugation the infranatant is exchanged with water and the washing is repeated. The functionality of the attached streptavidin is analysed by binding, e.g. to fluorescently labeled biotin, biotinylated antibodies (detected with a fluorescently labeled secondary antibody) or biotinylated and fluorescence- or radioactively-labeled oligonucleotides. Analysis is performed by fluorescence microscopy or scintillation counting.

d) Preparation of gas-filled microbubbles encapsulated with phosphatidylserine and a biotinylated oligonucleotide non-covalently bound to streptavidin-

succ-PEG_{3400}-DSPE

Microbubbles from (c) above are incubated in a solution containing a biotinylated oligonucleotide. The oligonucleotide-coated bubbles are washed as described above. Binding of the oligonucleotide to the bubbles is detected e.g. by using fluorescent-labeled oligonucleotides for attachment to the bubbles, or by hybridising the attached oligonucleotide to a labeled (fluorescence or radioactivity) complementary oligonucleotide. The functionality of the oligonucleotide-carrying microbubbles is analysed, e.g. by hybridising the bubbles with immobilized DNA-containing sequences complementary to the attached oligonucleotide. As examples, an oligonucleotide complementary to ribosomal DNA (of which there are many copies per haploid genome) and an oligonucleotide complementary to an oncogene (e.g. ras of which there is one copy per haploid genome) are used.

Example 6 - The peptide FNFRLKAGQKIRFGAAAWEPPRARI attached to gas-filled microbubbles encapsulated with phosphatidylserine

The peptide FNFRLKAGQKIRFGAAAWEPPRARI, comprising phosphatidylserine-binding and heparin-binding sections, is synthesised. The peptide is added to preformed phosphatidylserine-encapsulated perfluorobutane microbubbles and thoroughly mixed.

Example 7 - Gas-filled microbubbles encapsulated with phosphatidylserine and inactivated human thrombin-Succ-PEG_{3400}-DSPE

a) Inactivation of Human thrombin

Human thrombin is inactivated by incubation with a 20 %

molar excess of D-Phe-L-Pro-L-Arg-chloromethyl ketone in 0.05 M HEPES buffer, pH 8.0, at 37 °C for 30 minutes.

b) Preparation of gas-filled microbubbles encapsulated with phosphatidylserine and Succ-PEG_{3400}-DSPE

To a mixture (5 mg) of phosphatidylserine (90-99.9 mol%) and Succ-PEG_{3400}-DSPE (10 - 0.1 mol%, prepared as in Example 5(a)) is added 5% propyleneglycol-glycerol in water (1 ml). The dispersion is heated to not more than 80 °C for 5 minutes and is then cooled to ambient temperature. The dispersion (0.8 ml) is transferred to a vial (1 ml) and the head space is flushed with perfluorobutane. The vial is shaken in a cap-mixer for 45 seconds, whereafter the sample is put on a roller table. After centrifugation the infranatant is exchanged with water and the washing is repeated. Alternatively the microbubbles may be prepared as described in Preparation 1(f).

c) Preparation of gas-filled microbubbles encapsulated with phosphatidylserine and inactivated human thrombin-Succ-PEG_{3400}-DSPE

Inactivated human thrombin is covalently bound to succ-PEG_{3400}-DSPE in the microbubbles from (b) above by standard coupling methods using a water-soluble carbodiimide. The sample is placed on a roller table during the reaction. After centrifugation the infranatant is exchanged with water and the washing is repeated.

Example 8 - Gas-containing microparticles comprising polymer from ethylidene bis(16-hydroxyhexadecanoate) and adipoyl chloride and biotin-amidocaproate-Ala covalently attached to the polymer

a) Synthesis of Z-Ala-polymer (3-O-(carbobenzyloxy-L-alanyl)-polymer)

The polymer is prepared from ethylidene bis(16-hydroxyhexadecanoate) and adipoyl chloride as described in WO-A-9607434, and a polymer fraction with molecular weight 10000 is purified using gel permeation chromatography (GPC). 10 g of the material (corresponding to 1 mmol OH groups), Z-alanine (5 mmol) and dimethylaminopyridine (4 mmol) are dissolved in dry dimethylformamide/tetrahydrofuran and dicyclohexylcarbodiimide is then added. The reaction mixture is stirred at ambient temperature overnight. Dicyclohexylurea is filtered off and the solvent is removed using rotary evaporation. The product is purified by chromatography, fractions containing the *title compound* are combined and the solvent is removed using rotary evaporation. The structure of the product is confirmed by NMR.

b) Synthesis of Ala-polymer (3-O-(L-alanyl)-polymer)

Z-Ala-polymer (0.1 mmol) is stirred in toluene/tetrahydrofuran and glacial acetic acid (15% of the total volume) and hydrogenated in the presence of 5 % palladium on charcoal for 2 hours. The reaction mixture is filtered and concentrated *in vacuo*.

c) Synthesis of biotinamidocaproate-Ala-polymer

A solution of biotinamidocaproate N-hydroxysuccinimide ester in tetrahydrofuran is added to H_2N-Ala-polymer dissolved in a mixture of tetrahydrofuran and dimethylformamide and 0.1 M sodium phosphate buffer having a pH of 7.5. The reaction mixture is heated to 30 °C and stirred vigorously; the reaction is followed by TLC to completion. The solvent

is evaporated and the crude product is used without further purification.

d) Gas-containing particles comprising biotin-amidocaproate-Ala-polymer and PEG 10000 methyl ether 16-hexadecanoyloxyhexadecanoate

10 ml of a 5% w/w solution of biotin-amidocaproate-Ala-polymer in (-)-camphene maintained at 60 °C is added to 30 ml of an 1% w/w aqueous solution of PEG 10000 methyl ether 16-hexadecanoyloxyhexadecanoate (prepared as described in WO-A-9607434) at the same temperature. The mixture is emulsified using a rotor stator mixer (Ultra Turax® T25) at a slow speed for several minutes, and thereafter is frozen in a dry ice/methanol bath and lyophilized for 48 hours, giving the *title product* as a white powder.

e) Acoustic characterisation and microscopy of the product

Confirmation of the microparticulate nature of the product is performed using light microscopy as described in WO-A-9607434. Ultrasonic transmission measurements using a 3.5 MHz broadband transducer indicate that a particle suspension of < 2 mg/ml gives a sound beam attenuation of at least 5 dB/cm.

Example 9 - Functionalisation of of gas-filled albumin microspheres (GAM) with biotin

A homogeneous suspension of GAM ($6x10^8$ particles/ml) in 5 mg/ml albumin was used, with all manipulations being carried out at room temperature. Two 10 ml aliquots were centrifuged (170 x g, 5 minutes) to promote flotation of the microspheres and 8 ml of the underlying infranatant was removed by careful suction and replaced

by an equal volume of air-saturated phosphate buffered saline, the preparations being rotated for 15-20 minutes to resuspend the microspheres. This procedure was repeated twice, whereafter only negligible amounts of free non-microsphere-associated albumin were assumed to remain.

50 µl of NHS-biotin (10 mM in dimethylsulphoxide) was added to one of the aliquots (final concentration 50 µM); the other (control) aliquot received 50 µl of dimethylsulphoxide. The tubes containing the samples were rotated for 1 hour whereafter 20 µl portions of 50% aqueous glutaraldehyde were added to each tube to crosslink the microspheres. After rotation for another hour the tubes were positioned vertically overnight to allow flotation of the microspheres. The next day, the suspensions were washed twice with phosphate buffered saline containing 1 mg/ml human serum albumin (PBS/HSA) and were resuspended in PBS/HSA after the last centrifugation.

In order to determine the presence of microsphere-associated biotin, streptavidin conjugated to horseradish peroxidase (strep-HRP) was added to both suspensions and the tubes were rotated for 1 hour to allow for reaction. The microspheres were then washed three times, resuspended in 100 mM citrate-phosphate buffer (pH 5) containing 0.1 mg/ml phenylenediamine dihydrochloride and 0.01% hydrogen peroxide, and rotated for 10 minutes. Development of a yellow-green colour was indicative of the presence of enzyme. The following results were obtained:

Sample	Colour development
Biotinylated spheres + strp-HRP	2+

Control spheres + strp-HRP +

This confirms that GAM were biotinylated.

Example 10 - Method of use

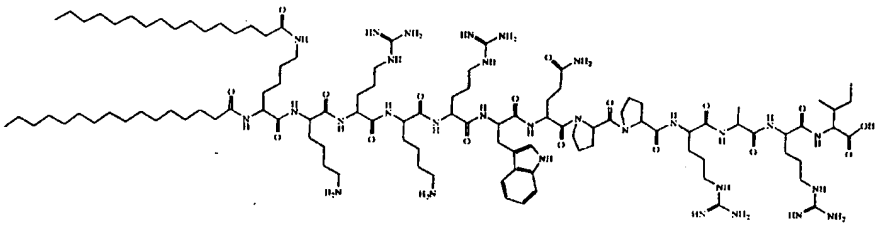
The agent from Example 7, comprising phosphatidylserine-encapsulated microbubbles having inactivated human thrombin-Succ-PEG_{3400}-DSPE incorporated into the encapsulating membrane is lyophilised from 0.01 M phosphate buffer, pH 7.4. The product is redispersed in sterile water and injected intravenously into a patient with suspected venous thrombosis in a leg vein. The leg is examined by standard ultrasound techniques. The thrombus is located by increased contrast as compared with surrounding tissue.

Example 11 - Adhesion of poly-L-lysine-coated phosphatidylserine-encapsulated microbubbles to endothelial cells

Poly-L-lysine (8 mg, Sigma P-1274 lot no. 14H5546) having a molecular weight of 115 kDa was dissolved in water (400 μl). Freshly redispersed microbubbles of phosphatidylserine-encapsulated perfluorobutane (40 μl) were incubated in either water (400 μl) or the poly-L-lysine solution for 15 minutes at room temperature. Zeta potential measurements confirmed that the poly-L-lysine-coated microbubbles were positively charged while the uncoated bubbles were negatively charged. A cell adhesion study using human endothelial cells grown in culture dishes (Type CRL 1730) was performed with the above-described microbubbles, the uncoated microbubbles being used as a control. Microscopy of the endothelial cells after incubation showed a much increased number of poly-L-lysine-coated microbubbles adhering to endothelial cells in comparison to the uncoated microbubbles.

Example 12 - Preparation and biological evaluation of gas-containing microbubbles of DSPS 'doped' with a lipopeptide consisting of a heparin sulphate binding peptide (KRKR) and a fibronectin peptide (WQPPRARI).

a) Synthesis of a lipopeptide consisting of a heparin sulphate binding peptide (KRKR) and fibronectin peptide (WQPPRARI).



The lipopeptide was synthesised on a ABI 433A automatic peptide synthesiser starting with Fmoc-Ile-Wang resin (Novabiochem) on a 0.1 mmol scale using 1 mmol amino acid cartridges. All amino acids and palmitic acid were preactivated using HBTU before coupling.
The simultaneous removal of peptide from the resin and side-chain protecting groups was carried out in TFA containing 5% phenol , 5% EDT, 5% anisole and 5% H_2O for 2 hours giving a crude product yield of 150 mg. Purification by preparative HPLC (Vydac 218TP1022 column) of a 40 mg aliquot of crude material was carried out using a gradient of 70 to 100 % B over 40 min (A= 0.1 % TFA/water and B = MeOH) at a flow rate of 9 mL/min. After lyophilization 16 mg of pure material was obtained (Analytical HPLC; Gradient, 70-100%B where B= MeOH, A= 0.01% TFA/water: column - vydac 218TP54: Detection - UV 260 and fluorescence, Ex_{280}, Em_{350} - product retention time = 19.44 min). Further product characterization was carried out using MALDI mass spectrometry; expected, M+H at 2198, found, at 2199.

b) Preparation of gas-containing microbubbles of DSPS 'doped' with a lipopeptide consisting of a heparin sulphate binding peptide (KRKR) and fibronectin peptide (WQPPRARI).

DSPS (Avanti, 4.5 mg) and lipopeptide from a) (0.5 mg) were weighed into each of 2 vials and 0.8 mL of a solution of 1.4% propylene glycol/2.4% glycerol was added to each vial. The mixture was warmed to 80°C for 5 minutes (vials shaken during warming). The samples were cooled to room temperature and the head space flushed with perfluorobutane gas. The vials were shaken in a cap mixer for 45 s and the microbubbles rolled overnight. Bubbles were washed several times with deionised water and analysed by Coulter counter (Size: 1-3 micron (87 %), 3-5 micron (11.5%)) and acoustic attenuation

(frequency max att.: 3.5 MHz). The microbubbles were stable at 120 mm Hg.

MALDI mass spectral analysis was used to confirm incorporation into DSPS microbubbles as follows; ca. 0.05-0.1 mL of microbubble suspension was transferred to a clean vial and 0.05-0.1 mL methanol added. The suspension was sonicated for 30 s and the solution analysed by MALDI MS. Positive mode gave M+H at 2200, expected for lipopeptide, 2198.

c) In vitro study of gas-containing microbubbles of DSPS 'doped' with a lipopeptide consisting of a heparin sulphate binding peptide (KRKR) and fibronectin peptide (WOPPRARI): binding to endothelial cells under flow conditions

The human endothelial cell line ECV 304, derived from a normal umbilical cord (ATCC CRL-1998) was cultured in 260 mL Nunc culture flasks (chutney 153732) in RPMI 1640 medium (Bio Whittaker) to which L-Glutamine 200 mM, Penicillin/ Streptomycin (10.000 U/mL and 10.000 mcg/mL) and 10% Fetal Bovine Serum (Hyclone Lot no. AFE 5183) were added.

The cells were subcultured with a split ratio of 1:5 to 1:7 when reaching confluence.

Cover-glasses, 22mm in diameter (BDH, Cat no. 406/0189/40) were sterilised and placed on the bottom of 12 well culture plates (Costar) before cells in 0,5 mL complete medium with serum was added on top.

When the cells reached confluence the coverslips were placed in a custom made flow-chamber. The chamber consists of a groove carved into a glass plate upon which the cover slip with cells was placed with the cells facing the groove forming a flow channel.

Ultrasound microbubbles from section b) were passed from a reservoir held at 37 degree Celsius through the flow chamber and back to the reservoir using a peristaltic

pump. The flow rate was adjusted to simulate physiological relevant shear rates. The flow chamber was placed under a microscope and the interaction between the microspheres and cells viewed directly. A camera mounted on the microscope was connected to a colour video printer and a monitor.

A gradual accumulation of the microbubbles on the cells took place which was dependant on the flow rate. By increasing the flow rate the cells started to become detached from the coverslip, the microbubbles were still bound to the cells. Control bubbles not carrying the vector did not adhere to the endothelial cells and disappeared from the cells under minimal flow conditions.

d) In vivo experiment in dog

Case 1)

A 22 kg mongrel dog was anaesthetized with pentobarbital and mechanically ventilated. The chest was opened by a midline sternotomy, the anterior pericardium was removed, and a 30 mm gelled silicone rubber spacer was inserted between the heart and a P5-3 transducer of an ATL HDI-3000 ultrasound scanner. The scanner was set for intermittent short axis imaging once in each end-systole by delayed EGC triggering.

A net volume of 2 mL of microbubbles from b) were injected as a rapid intravenous bolus. 3 seconds later, the imaged right ventricle was seen to contain contrast material, another 3 seconds later, the left ventricle was also filled, and a transient attenuation shadow that obscured the view of the posterior parts of the left ventricle was observed. A substantial increase in brightness of the myocardium was seen, also in the portions of the heart distal to the left ventricle when the attenuation shadow subsided.

After passage of the inital bolus, the ultrasound scanner was set to continuous, high frame rate high output power imaging, a procedure known to cause destruction of utrasound contrast agent bubbles in the imaged tissue regions. After a few seconds, the scanner was adjusted back to its initial setting. The myocardium was then darker, and closer to the baseline value. Moving the imaged slice to a new position resulted in re-appearance of contrast effects, moving the slice back to the initial position again resulted in a tissue brightness again close to baseline.

Case 2) [comparative]

A net volume of 2 mL microbubbles prepared in an identical manner to b) above with the exception that no lipopeptide was included in the preparation was injected, using the same imaging procedure as above. The myocardial echo enhancement was far less intense and of shorter duration than observed in case 1. At the completion of the left ventricular attenuation phase, there was also almost complete loss of myocardial contrast effects, and a myocardial echo increases in the posterior part of the left ventricle as in case 1 was not observed.

Example 13 - Targeted gas-containing microbubbles of DSPS coated non-covalently with polylysine and a fusion peptide comprising a PS binding component and a Fibronectin peptide sequence
NH_2F.N.F.R.L.K.A.G.O.K.I.R.F.G.G.G.G.W.O.P.P.R.A.I.OH.

a) Synthesis of PS binding/Fibronectin fragment fusion peptide
NH_2F.N.F.R.L.K.A.G.O.K.I.R.F.G.G.G.G.W.O.P.P.R.A.I.OH.

The peptide was synthesised on an ABI 433A automatic

peptide synthesiser starting with Fmoc-Ile-Wang resin (Novabiochem) on a 0.1 mmol scale using 1 mmol amino acid cartridges. All amino acids were preactivated using HBTU before coupling.

The simultaneous removal of peptide from the resin and side-chain protecting groups was carried out in TFA containing 5% phenol , 5% EDT and 5% H_2O for 2 hours giving a crude product yield of 302 mg. Purification by preparative HPLC (Vydac 218TP1022 column) of a 25 mg aliquot of crude material was carried out using a gradient of 20 to 40 % B over 40 min (A= 0.1 % TFA/water and B = 0.1 % TFA/acetonitrile) at a flow rate of 9 mL/min. After lyophilization 10 mg of pure material was obtained (Analytical HPLC; Gradient, 20 to 50% B where B= 0.1% TFA/acetonitrile, A= 0.01% TFA/water: column - vydac 218TP54: Detection - UV 214 and 260 nm - product retention time = 12.4 min). Further product characterization was carried out using MALDI mass spectrometry; expected, M+H at 2856, found, at 2866.

b) Preparation of microbubbles of DSPS coated non-covalently with polylysine and the PS binding/Fibronectin fragment fusion peptide NH_2F.N.F.R.L.K.A.G.O.K.I.R.F.G.G.G.G.W.O.P.P.R.A.I.OH.

DSPS (5 mg, Avanti) was weighed into a clean vial along with poly-L-lysine (Sigma, 0.2 mg) and peptide from a) above (0.2 mg). To the vial was added 1.0 mL of a solution of 1.4% propylene glycol/ 2.4% glycerol. The mixture was warmed to 80°C for 5 minutes. The sample was cooled to room temperature and the head space flushed with perfluorobutane gas. The vials were shaken in a cap mixer for 45 s and the microbubbles centrifuged at 1000 rpm for 3 minutes.

Following extensive washing with water, PBS and water the final solution was examined for polylysine and peptide content using MALDI MS. No polypeptide material

was observed in the final wash solution. Acetonitrile (0.5 mL) was then added and the microbubbles destroyed by sonication. Analysis of the resulting solution for polylysine and PS-binding/fibronectin fusion peptide was then carried out using MALDI MS. The results were as follows:

	MALDI expected	MALDI found
Poly-L-lysine	786, 914, 1042, 1170	790, 919, 1048, 1177
DSPS-binding peptide	2856	2866

The spacer element contained within the PS binding/Fibronectin fusion peptide (-GGG-) can also be replaced with other spacers such as PEG_{2000} or poly alanine (-AAA-). It is also envisaged that a form of pre-targeting may be employed, whereby the DSPS binding/Fibronectin fragment fusion peptide is firstly allowed to associate with cells via the fibronectin peptide binding. This is followed by administration of PS microbubbles which then bind to the PS binding peptide.

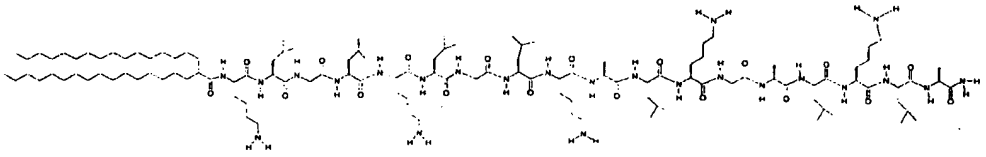
Example 14 - Gas containing microbubbles of DSPS covalently modified with CD71 FITC-labelled anti-transferrin receptor antibody and 'doped' with a lipopeptide with affinity for endothelial cells.

This example is directed at the preparation of multiple vector targeted ultrasound agents.

a) Synthesis of an endothelial cell binding lipopeptide: 2-n-hexadecylstearyl-Lys-Leu-Ala-Leu-Lys-Leu-Ala-Leu-Lys-Ala-Leu-Lys-Ala-Ala-Leu-Lys-Leu-Ala-NH_2.

The lipopeptide shown below was synthesised on a ABI 433A automatic peptide synthesiser starting with a Rink

amide resin on a 0.1 mmol scale using 1 mmol amino acid cartridges.



All amino acids and 2-n-hexadecylstearic acid were preactivated using HBTU before coupling. The simultaneous removal of peptide from the resin and side-chain protecting groups was carried out in TFA containing 5% EDT, and 5% H_2O for 2 hours giving a crude product yield of 150 mg. Purification by preparative HPLC (Vydac 218TP1022 column) of a 40 mg aliquot of crude material was carried out using a gradient of 90 to 100 % B over 50 min (A= 0.1 % TFA/water and B = MeOH) at a flow rate of 9 mL/min. After lyophilization 10 mg of pure material was obtained (Analytical HPLC; Gradient, 90-100%B where B= MeOH, A= 0.01% TFA/water: column - vydac 218TP54: Detection - UV 214 nm - product retention time = 23 min). Further product characterization was carried out using MALDI mass spectrometry; expected, M+H at 2369, found, at 2373.

b) Preparation of gas-containing microbubbles of DSPS 'doped' with a endothelial cell binding lipopeptide and PE-PEG_{2000}-MAL

DSPS (Avanti, 4.5 mg) and lipopeptide from a) (0.5 mg) along with PE-PEG_{2000}-Maleimide from example 2 (0.5 mg) were weighed into a clean vial and 1 mL of a solution of 1.4% propylene glycol/2.4% glycerol added. The mixture was warmed to 80°C for 5 minutes then filtered through a 4.5 micron filter. The sample was cooled to room temperature and the head space flushed with

perfluorobutane gas. The vials were shaken in a cap mixer for 45 s and the microbubbles washed three times with distilled water.

c) Thiolation of FITC-labelled anti-transferrin receptor antibody.

FITC labelled CD71 anti-transferrin receptor Ab (100 μg/mL, Becton Dickinson), 0.7 mL, in PBS was modified with Traut's reagent (0.9 mg, Pierce) at room temperature for 1 h. Excess reagent was separated from modified protein on a NAP-5 column (Pharmacia).

d) Conjugation of thiolated FITC-labelled anti-transferrin receptor antibody to gas-containing microbubbles of DSPS 'doped' with an endothelial cell binding lipopeptide and DSPE-PEG_{2000}-MAL

A 0.5 mL aliquot of the protein fraction (2 mL in total) from c) above was added to the microbubbles from b) and the conjugation reaction allowed to proceed for 10 min on a roller table. Following centrifugation at 1000 rpm for 3 min the protein solution was removed and the conjugation repeated a further two times with 1 mL and 0.5 mL aliquots of protein solution respectively. The bubbles were then washed four times in distilled water and a sample analysed for the presence of antibody by flow cytometry and microscopy. A fluorescent population of >92% was observed.

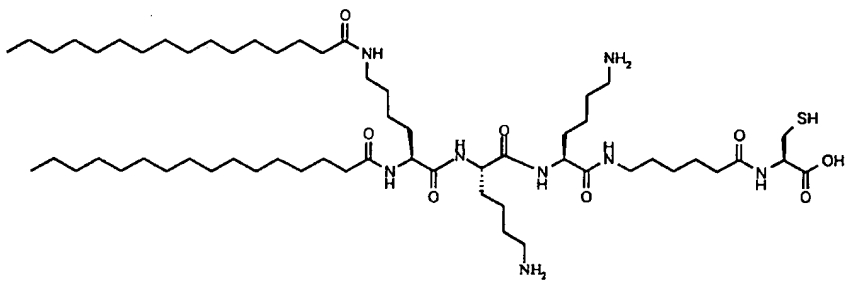
Fig. 1 of the accompanying drawing represents the flow cytometric comparison of negative control microbubbles of DSPS (left curve) with bubbles conjugated with CD71 FITC-labelled anti-transferrin antibody (filled curve, right), showing that 92% of the population fluoresce.

Incorporation into the microbubbles of lipopeptide was confirmed by MALDI mass spectrometry as described in example 12 b).

Example 15 - Preparation of Transferrin/Avidin coated gas-filled microbubbles for targeted ultrasound imaging.

This example is directed to the preparation of microbubbles containing multiple protein vectors for targeted ultrasound/therapy.

a) Synthesis of a thiol functionalised lipid molecule: Dipalmitoyl-Lys-Lys-Lys-Aca-Cys.OH



The lipid structure shown above was synthesised on a ABI 433A automatic peptide synthesiser starting with Fmoc-Cys(Trt)-Wang resin (Novabiochem) on a 0.25 mmol scale using 1 mmol amino acid cartridges. All amino acids and palmitic acid were preactivated using HBTU coupling chemistry.

The simultaneous removal of peptide from the resin and deprotection of side-chain protecting groups was carried out in TFA containing 5% EDT, and 5% H_2O for 2 hours giving a crude product yield of 250 mg. Purification by preparative HPLC (Vydac 218TP1022 column) of a 40 mg aliquot of crude material was carried out using a gradient of 90 to 100 % B over 50 min (A= 0.1 % TFA/water and B = MeOH) at a flow rate of 9 mL/min. After lyophilization 24 mg of pure material was obtained (Analytical HPLC; Gradient, 70-100%B where B= 0.1% TFA/ acetonitrile, A=

0.01% TFA/water: column - vydac 218TP54: Detection - UV 214 nm-product retention time = 23 min). Further product characterization was carried out using MALDI mass spectrometry; expected, M+H at 1096, found, at 1099.

b) Preparation of gas-containing microbubbles of DSPS 'doped' with a thiol containing lipid structure:

DSPS (Avanti, 4.5 mg) and the lipid structure from a) above (0.5 mg) were weighed into a clean vial and 0.8 mL of a solution containing 1.4% propylene glycol/ 2.4% glycerol in water added. The mixture was warmed to 80°C for 5 minutes (vials shaken during warming) and filtered while still hot through a 40 micron filter. The samples were cooled to room temperature and the head space flushed with perfluorobutane gas. The vials were shaken in a cap mixer for 45 s and the microbubbles placed on roller table overnight. Bubbles were washed several times with deionised water and analysed for thiol group incorporation using Ellmans Reagent.

c) Modification of transferrin and avidin with Fluorescein-NHS and Sulpho-SMPB.

To a mixture of 2 mg of transferrin (Holo, human, Alpha Therapeutic Corp) and 2 mg of avidin (Sigma) in PBS (1 mL) was added 0.5 mL DMSO solution containing 1 mg Sulpho-SMPB (Pierce) and 0.5 mg Fluorescein-NHS (Pierce). The mixture was stirred for 45 minutes at room temperature then passed through a Sephadex 200 column using PBS as eluent. The protein fraction was collected and stored at 4°C prior to use.

d) Microbubble conjugation with modified Transferrin/Avidin.

To the thiol containing microbubbles from b) was added 1

mL of the modified transferrin/avidin protein solution c). After adjusting the pH of the solution to 9 the conjugation reaction was allowed to proceed for 2 h at room temperature. Following extensive washing with deionised water the microbubbles were analysed by Coulter counter (81% between 1 and 7 micron) and fluorescence microscopy (highly fluorescent microbubbles were observed).

Example 16 - Preparation of functionalised gas-filled microbubbles for targeted ultrasound imaging.

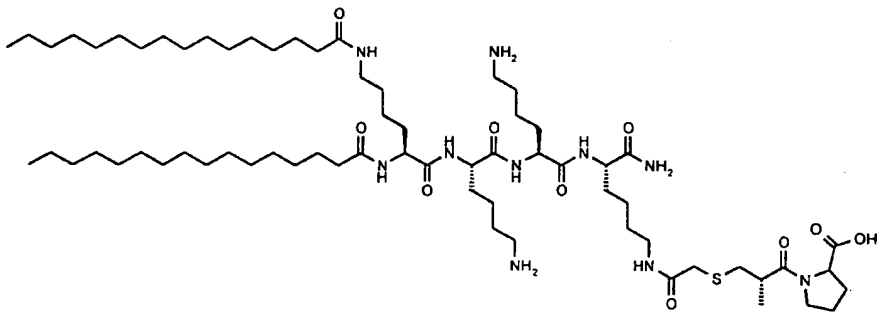
This example is directed to the preparation of microbubbles having a reactive group on the surface for non-specific targeting, principally utilising disulphide exchange reactions to effect binding to a multiplicity of cellular targets.

DSPS (Avanti, 5.0 mg) and the thiol containing lipid structure from example 15 a)(1.0 mg) were weighed into a clean vial and 0.8 mL of a solution containing 1.4% propylene glycol/ 2.4% glycerol in water added. The mixture was warmed to 80°C for 5 minutes (vials shaken during warming) and filtered while still hot through a 40 micron filter. The samples were cooled to room temperature and the head space flushed with perfluorobutane gas. The vials were shaken in a cap mixer for 45 s and the microbubbles placed on roller table overnight. Bubbles were washed several times with deionised water and analysed for thiol group incorporation using Ellmans Reagent.

Example 17 - Gas-containing microbubbles of DSPS comprising a lipopeptide for endothelial cell targeting and a captopril containing molecule.

This example is directed to the preparation of ultrasound agents for combined targeting and therapeutic applications.

a) Synthesis of a lipopeptide functionalised with captopril:

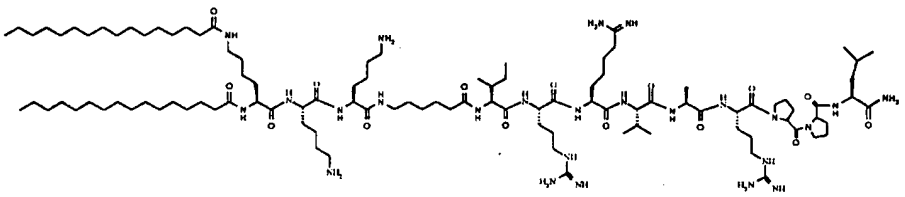


The structure shown above was synthesised using a manual nitrogen bubbler apparatus starting with Fmoc protected Rink Amide MBHA resin (Novabiochem) on a 0.125 mmol scale. All amino acids were purchased from Novabiochem and palmitic acid from Fluka. Coupling was carried out using standard TBTU/HOBt/DIEA protocols. Bromoacetic acid was coupled through the side-chain of Lys as a symmetrical anhydride using DIC preactivation. Captopril (Sigma) dissolved in DMF was introduced on the solid-phase using DBU as base.

Simultaneous removal of the peptide from the resin and deprotection of side-chain protecting groups was carried out in TFA containing 5% EDT, 5% water and 5% ethyl methyl sulphide for 2 h. An aliquot of 10 mg of the crude material was purified by preparative liquid chromatography (Vydac 218TP1022 column) using a gradient of 70 to 100% B over 60 min (A = 0.1% TFA/water and B = 0.1% TFA/acetonitrile) at a flow rate of 10 mL/min. After lyophilization a yield of 2 mg of pure material was obtained (analytical HPLC: gradient 70-100% B over 20 min, A = 0.1% TFA/water and B = 0.1% TFA/acetonitrile; flow rate 1 mL/min; column Vydac

218TP54; detection UV 214 nm; retention time 26 min). Further characterisation was carried out using MALDI mass spectrometry, giving M+H at 1265 as expected.

b) Synthesis of a lipopeptide with affinity for endothelial cells: Dipalmitoyl-Lys-Lys-Lys-Aca-Ile-Arg-Arg-Val-Ala-Arg-Pro-Pro-Leu-NH_2



The lipopeptide was synthesised on a ABI 433A automatic peptide synthesiser starting with Rink amide resin (Novabiochem) on a 0.1 mmol scale using 1 mmol amino acid cartridges. All amino acids and palmitic acid were preactivated using HBTU before coupling.
The simultaneous removal of peptide from the resin and side-chain protecting groups was carried out in TFA containing 5% phenol , 5% EDT and 5% H_20 for 2 hours giving a crude product yield of 160 mg. Purification by preparative HPLC (Vydac 218TP1022 column) of a 35 mg aliquot of crude material was carried out using a gradient of 70 to 100 % B over 40 min (A= 0.1 % TFA/water and B = MeOH) at a flow rate of 9 mL/min. After lyophilization 20 mg of pure material was obtained (Analytical HPLC; Gradient, 70-100%B where B= MeOH, A= 0.01% TFA/water: column - vydac 218TP54: Detection - UV 214 and 260 nm - product retention time = 16 min). Further product characterization was carried out using MALDI mass spectrometry; expected, M+H at 2198, found, at 2199.

c) Preparation of gas-containing microbubbles of DSPS comprising a lipopeptide for endothelial cell targeting

and a captopril containing molecule for drug delivery

DSPS (Avanti, 4.5 mg), product from a) (0.5 mg) and product from b) (0.5 mg) were weighed into a vial and 1.0 mL of a solution of 1.4% propylene glycol/ 2.4% glycerol was added to each vial. The mixture was warmed to 80°C for 5 minutes (vials shaken during warming). The samples were cooled to room temperature and the head space flushed with perfluorobutane gas. The vials were firstly shaken in a cap mixer for 45 s then rolled for 1 h followed by extensive washing with deionised water. No detectable levels of starting material was found in the final wash solution as evidenced by MALDI MS.
MALDI mass spectral analysis was used to confirm incorporation of the products from section a) and b) into the microbubbles as described in example 12 b).

Example 18 - Preparation of gas-containing microbubbles of DSPS loaded with a lipopeptide comprising a helical peptide with affinity for cell membranes and the peptide antibiotic polymixin B sulphate.

This example is directed at the preparation of targeted microbubbles comprising multiple peptidic vectors having a combined targeting and a therapeutic application.

a) Synthesis of a lipopeptide comprising a helical peptide with affinity for cell membranes:hexadecylstearyl-Lys-Leu-Ala-Leu-Lys-Leu-Ala-Leu-Lys-Ala-Leu-Lys-Ala-Ala-Leu-Lys-Leu-Ala-NH_2.

Described in example 14 a).

b)- Preparation of multiple-specific gas-containing microbubbles.

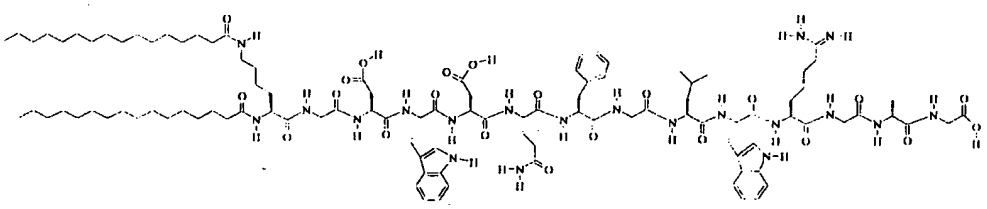
DSPS (Avanti, 5.0 mg), lipopeptide from a)(0.3 mg)and

polymixin B sulphate (Sigma, 0.5 mg) was weighed into a clean vial and 1.0 mL of a solution of 1.4% propylene glycol/ 2.4% glycerol added. The mixture was sonicated for 3-5 mins, warmed to 80°C for 5 minutes then filtered through a 4.5 micron filter. The mixture was cooled to room temperature and the head space flushed with perfluorobutane gas. The vial was shaken in a cap mixer for 45 s and the microbubbles centrifuged at 1000 rpm for 3 minutes. The microbubbles were washed in water until no polymixin B sulphate or lipopeptide could be detected in the infranatant by MALDI-MS. Microscopy showed that the size distribution of the bubble population was between 1-8 micron as desired.
To the washed bubbles (ca. 0.2 mL) was added methanol (0.5 mL) and the mixture placed in a sonic bath for 2 min. The resulting clear solution, following analysis by MALDI-MS, was found to contain both lipopeptide and polymixin B sulphate (expected 1203, found 1207).

Example 19 - Preparation of multiple-specific gas-containing microbubbles of DSPS 'doped' with a lipopeptide comprising a IL-1 receptor binding sequence and modified with a branched structure containing the drug methotrexate.

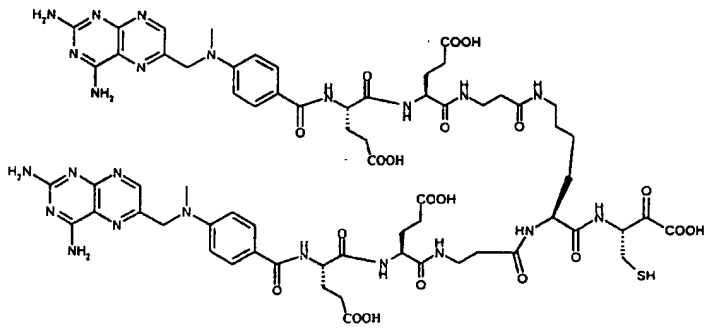
This example is directed at the preparation of targeted microbubbles comprising a non-bioactive vector for targeting and a component for drug delivery.

a) Synthesis of a lipopeptide comprising an interleukin-1 receptor binding peptide: Dipalmitoyl-Lys-Gly-Asp-Trp-Asp-Gln-Phe-Gly-Leu-Trp-Arg-Gly-Ala-Ala.OH



The lipopeptide was synthesised on a ABI 433A automatic peptide synthesiser starting with Fmoc-Ala-Wang resin (Novabiochem) on a 0.1 mmol scale using 1 mmol amino acid cartridges. All amino acids and palmitic acid were preactivated using HBTU before coupling.
The simultaneous removal of lipopeptide from the resin and side-chain protecting groups was carried out in TFA containing 5% H_2O, 5% anisole, 5 % phenol and 5% EDT for 2 hours giving a crude product yield of 150 mg.
Purification by preparative HPLC (Vydac 218TP1022 column) of a 30 mg aliquot of crude material was carried out using a gradient of 90 to 100 % B over 40 min (A= 0.1 % TFA/water and B = MeOH) at a flow rate of 9 mL/min. After lyophilization 4 mg of pure material was obtained (Analytical HPLC; Gradient, 90-100%B over 20 min where B= MeOH, A= 0.01% TFA/water: column - vydac 218TP54: Detection - UV 214 nm; product retention time = 23 min). Further product characterization was carried out using MALDI mass spectrometry; expected, M+H at 2083, found, at 2088.

b) Synthesis of a branched methotrexate core structure containing a thiol moiety.



The methotrexate structure was synthesised on a ABI 433A automatic peptide synthesiser starting with Fmoc-Cys(Trt) Tentagel resin on a 0.1 mmol scale.
The simultaneous removal of product from the resin and

deprotection of protecting groups was carried out in TFA containing 5% EDT and 5% H_2O for 2 hours giving a crude product yield of 160 mg. Purification by preparative HPLC (Vydac 218TP1022 column) of a 30 mg aliquot of crude material was carried out using a gradient of 10 to 30 % B over 40 min (A= 0.1 % TFA/water and B = 0.1 % TFA/acetonitrile) and a flow rate of 9 mL/min. After lyophilization of the pure fractions 9 mg of pure material was obtained (Analytical HPLC; Gradient, 5-50 %B where B = 0.1 % TFA/acetonitrile, A= 0.01% TFA/water: column - vydac 218TP54: Detection - UV 214 nm - product retention time = 9.5 min). Further product characterization was carried out using MALDI mass spectrometry; expected, M+H at 1523, found, 1523.

c)- Preparation of multiple-specific gas-containing microbubbles.

DSPS (Avanti, 4.5 mg) and thiol containing lipopeptide from example 15 a) (0.5 mg) and lipopeptide from a) (0.2 mg) above were weighed into a clean vial and 1.0 mL of a solution of 1.4% propylene glycol/ 2.4% glycerol added. The mixture was sonicated for 3-5 mins, warmed to 80°C for 5 minutes then filtered through a 4.5 micron filter. The mixture was cooled to room temperature and the head space flushed with perfluorobutane gas. The vials were shaken in a cap mixer for 45 s and the microbubbles centrifuged at 1000 rpm for 3 minutes following which the infranatant was discarded.

d) Conjugation of methotrexate branched structure to thiolated microbubbles.

The methotrexate structure from b) above (0.5 mg) was dissolved in PBS pH 8.0. The solution was then added to the thiol containing bubbles from c) and disulphide bond formation allowed to proceed for 16 h. Following

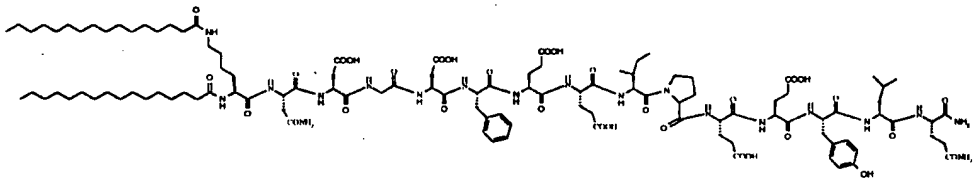
extensive washing with PBS and water the bubbles were analysed by microscopy and MALDI MS.

It is also considered relevant that the disulphide bond linking the methotrexate structure to the microbubble may be reduced in vivo liberating the free drug molecule. This in combination with a tumour specific vector is a drug delivery system. A physiologically relevant reducing agent such as glutathione may be used to bring about drug release.

Example 20 - Preparation of microbubbles encapsulated with DSPS and functionalised with a thrombi-targeting lipopeptide and the thrombolytic enzyme tissue plasminogen activator.

This example is directed at the preparation of thrombus targeted US agents comprising a therapeutic thromolytic agent.

a) Synthesis of a lipopeptide with affinity for thrombi (Diplamitoyl-Lys-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln.NH_2).



The lipopeptide was synthesised on a ABI 433 A automatic peptide synthesiser starting with Rink amide resin (Novabiochem) on a 0.1 mmol scale using 1 mmol amino acid cartridges. All amino acids and palmitic acid were preactivated using HBTU before coupling.
The simultaneous removal of peptide from the resin and side-chain protecting groups was carried out in TFA

containing 5% phenol, 5% EDT, 5% anisole and 5% H_2O for 2 h giving a crude product yield of 80 mg. Purification by preparative HPLC (Vydac 218TP1022 column) of a 20 mg aliquot of the crude material was carried out. After lyophilization 6 mg of pure material was obtained. The product was characterized by MALDI mass spectrometry and analytical HPLC.

b) Modification of tissue plasminogen activator with Sulpho-SMPB.

A solution of 0.1 mL of ammonim carbonate buffer containing 0.1 mg of t-PA (Sigma) was made up to 0.2 mL by the addition of water. To this solution was added 0.4 mg of Sulpho-SMPB (Pierce) dissolved in 0.05 mL DMSO. The protein solution was left standing at room temperature for 45 min then purification carried out on a Superdex 200 column. The product was eluted in PBS and the modified protein fraction collected.

c) Preparation of microbubbles encapsulated with DSPS/thrombi-binding lipopeptide and thiol containing lipoeptide and conjugation of modified tissue plasminogen activator.

DSPS (Avanti, 5.0 mg) was weighed into a clean vial along with 0.5 mg of the lipopeptide from a) and 0.5 mg of the thiol containing lipopeptide from example 15 a). To this was added 1.0 mL of a solution of 1.4% propylene glycol/ 2.4% glycerol and the mixture sonicated for 2 min then warmed to 80°C for 5 minutes. Immediately following warming the solution was filtered through a 4 micron filter. The sample was cooled to room temperature and the head space flushed with perfluorobutane gas. The vials were shaken in a cap mixer for 45 s and the microbubbles washed 2 times with deionised water. The infranatant was discarded and replaced with a 1 mL

aliquot of the protein solution from b) above. The conjugation reaction was allowed to proceed for 1 h. The bubbles were centrifuged and infranatant exchanged with a further 1 mL of protein solution. The incubation step was repeated until all protein solution was used up. The microbubbles were then washed extensively with water and analysed by Coulter counter. The microbubbles were tested in the flow chamber assay described in example 12 c). Microbubbles modified with protein were found to bind in higher numbers than those comprising either lipopeptide/DSPS or DSPS alone.

It is envisaged that the targeting/therapeutic/ultrasound activities of these microbubbles be evaluated in models of in vitro and in vivo thrombogenisis.

Example 21 - Preparation of gas-containing microbubbles encapsuled with DSPS comprising thiolated anti-CD34-MAL-PEG_{2000}-PE

a) Preparation of gas containing microbubbles encapsuled with DSPS and PE-PEG 2000-Mal

DSPS (Avanti, 4,5 mg) and PE-PEG_{2000}-maleimide from example x (0,5 mg) were weighed into a clean vial and 1 mL of a solution of 1.4% propylene glyco/2.4% glycerol added. The mixture was warmed to 80°C for 5 minutes then filtered through a 4.5 micron filter. The sample was cooled to room temperature and the head space flushed with perfluorbutane gas. The vials were shaken in a cap mixer for 45 s and the microbubbles washed three times with destilled water.

b) Thiolation of anti-CD34 antibodies.

To 0.3 mg of anti-CD34 antibody dissolved in 0.5 mL PBS buffer pH7,was added 0.3 mg Traut's reagent and the solution stirred at room temperature for 1 h. Excess reagent was separated from the modified protein on a NAP-5 column (Pharmacia).

c) Conjugation of thiolated anti-CD34 antibody to gas-containing microbubbles encapsuled with DSPS and comprising DSPE-PEG $_{2000}$-MAL

0.5mL of the thiolated antibody praparation from b) was added to an aliqout of microbubbles from a) and the conjugation reaction allowed to proceed for 30 min on a roller table. Following centifugation at 2000 rpm for 5 min the infranatant was removed. The microbubbles were washed a further three times with water.

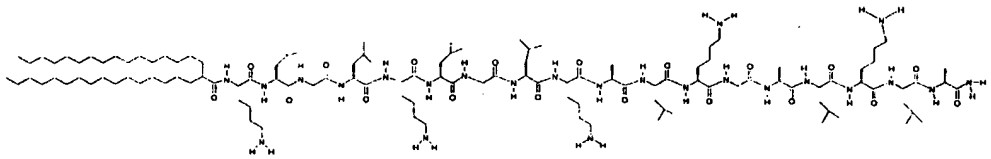
d) Detection of the antibody encapsuled in the microbubbles using a FITC-conjugated secondary antibody.

To the microbubbles suspension from c) was added 0.025 mL FITC-conjugated goat-anti-mouse antibody. The mixture was incubated at room temperature (dark) for 30 min on a roller table then centrifugation at 2000 rpm for 5 min. The infranatant was then removed and the microbubbles washed a further three times with water. Flow cytometric analysis of the microbubble suspension showed that 98% of the population were fluorescent.

Example 22 - Preparation of gas-containing microbubbles of DSPS loaded with a lipopeptide comprising a helical peptide with affinity for cell membranes

This example is directed at the preparation of targeted microbubbles comprising a non-bioactive peptidic vector for targeting of cell membrane structures.

a) Synthesis of a lipopeptide comprising a helical peptide with affinity for cell membranes



The lipopeptide was synthesised on a ABI 433A automatic peptide synthesiser starting with Rink amide resin (Novabiochem) on a 0.2 mmol scale using 1mmol amino acid cartridges. All amino acids and 2-n-hexadecylstearic acid were preactivated using HBTU before coupling. The simultaneous removal of lipopeptide from the resin and side-chain protecting groups was carried out in TFA containing 5% H_2O for 2 hours giving a crude product yield of 520 mg. Purification by preparative HPLC (Vydac 218TP1022 column) of a 30 mg aliqout of crude material was carried out using a gradient of 90 to 100 % B over 40 min (A= 0.1 % TFA/water and B = MeOH) at a flow rate of 9 ml/min. After lyophilization 10 mg of pure material was obtained (Analytical HPLC; Gradient, 90-100%B over 20 min where B= MeOH, A= 0.01% TFA/water: column - vydac 218TP54: Detection - UV 214 nm; product retention time = 23 min). Further product characterization was carried out using MALDI mass spectrometry; expected, M+H at 2369, found, at 2375.

b) - Preparation of gas-containing microbubbles.

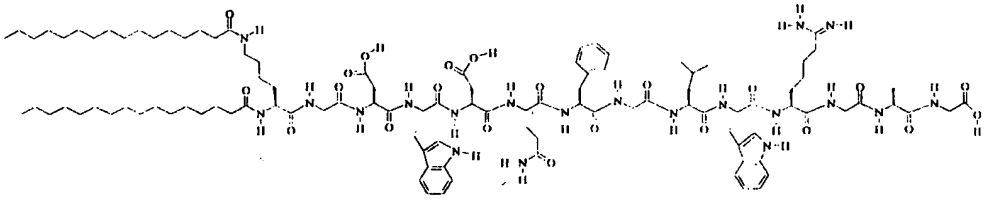
DSPS (Avanti, 4.5 mg) and lipopeptide from a) (0.5 mg) was weighed into a clean vial and 1.0 ml of a solution of 1.4% propylene glycol/ 2.4% glycerol added. The mixture was sonicated for 3-5 mins, warmed to 80°C for 5 minutes then filtered through a 4.5 mm filter. The mixture was cooled to room temperature and the head

space flushed with perfluorobutane gas. The vials were shaken in a cap mixer for 45 s and the microbubbles centrifuged at 1000 rpm for 3 minutes. The bubbles were washed in water until no lipopeptide could be detected by MALDI-MS. Coulter counter, acoustic attenuation and pressure stability studies were performed.
To an aliquot of the washed bubbles (ca. 0.2 mL) was added methanol (0.5 mL) and the mixture placed in a sonic bath for 2 min. The resulting clear solution, following analysis by MALDI-MS, was found to contain the lipopeptide. The microbubbles had similar characteristics in vitro and in vivo as was found for the microbubbles made in example 12.

Example 23 - Preparation of gas-containing microbubbles of DSPS loaded with a lipopeptide comprising a non-bioactive interleukin 1 receptor binding peptide

This example is directed at the preparation of targeted microbubbles comprising a non-bioactive peptidic vector for targeting at the IL-1 recptor which does not induce signal tranduction or prevent IL-1 binding.

a) Synthesis of a lipopeptide comprising a non-bioactive interleukin 1 receptor binding peptide



The lipopeptide was synthesised on a ABI 433A automatic peptide synthesiser starting with Fmoc-Ala-Wang resin (Novabiochem) on a 0.1 mmol scale using 1 mmol amino

acid cartridges. All amino acids and palmitic acid were preactivated using HBTU before coupling.
The simultaneous removal of lipopeptide from the resin and side-chain protecting groups was carried out in TFA containing 5% H_2O, 5% anisole, 5 % phenol and 5% EDT for 2 hours giving a crude product yield of 150 mg. Purification by preparative HPLC (Vydac 218TP1022 column) of a 30 mg aliqout of crude material was carried out using a gradient of 90 to 100 % B over 40 min (A= 0.1 % TFA/water and B = MeOH) at a flow rate of 9 ml/min. After lyophilization 4 mg of pure material was obtained (Analytical HPLC; Gradient, 90-100%B over 20 min where B= MeOH, A= 0.01% TFA/water: column - vydac 218TP54: Detection - UV 214 nm; product retention time = 23 min). Further product characterization was carried out using MALDI mass spectrometry; expected, M+H at 2083, found, at 2088.

b) - Preparation of multiple-specific gas-containing microbubbles.

DSPS (Avanti, 4.5 mg) and lipopeptide from a)(0.5 mg) were weighed into a clean vial and 1.0 ml of a solution of 1.4% propylene glycol/ 2.4% glycerol added. The mixture was sonicated for 3-5 mins, warmed to 80°C for 5 minutes then filtered through a 4.5 micron filter. The mixture was cooled to room temperature and the head space flushed with perfluorobutane gas. The vials were shaken in a cap mixer for 45 s and the microbubbles centrifuged at 1000 rpm for 3 minutes. The bubbles were washed in water until no lipopeptide could be detected by MALDI-MS.
To the washed bubbles (ca. 0.2 mL) was added methanol (0.5 mL) and the mixture placed in a sonic bath for 2 min. The resulting clear solution, following analysis by MALDI-MS, was found to contain lipopeptide (expected

2083, found 2088).

Example 24 - Preparation of PFP containing microbubbles of DSPC, DSPS and endothelial cell binding lipopeptide for targeted ultrasound imaging.

To 0.8 ml of a solution containing DSPC:DSPS (3:1) (5mg/ ml) in propyleneglycol/glycerol (4% in water) was added 0.5 mg of the lipopeptide from example 17 b). The mixture was heated to 80°C for 5 minutes and shaken. The solution was then cooled to ambient temperature and the headspace was flushed with perfluoropropane. The vial was shaken on a Capmix (Espe Capmix) for 45 seconds and placed on a roller table for 5 min. The sample was centrifuged (Juan MR 14.11) at 2000 rpm for 5 minutes and the infranatant removed and replaced with destilled water. The headspace was again flushed with perfluorobutane and the sample was kept on a table roller until a homogenous appearance was obtained. The washing procedure was repeated. The resulting ultrasound contrast agent was confirmed by Coulter counter, acoustic attenuation measurements and resistance to external pressure.
The microbubbles were tested in the in vitro assay as detailed in example 12. A gradual accumulation of bubbles binding to the cells was observed.

Example 25 - Preparation of SF_6 containing microbubbles of DSPC, DSPS and endothelial cell binding lipopeptide for targeted ultrasound imaging.

To 0.8 ml of a solution containing DSPC:DSPS (3:1) (5mg/ ml) in propyleneglycol/glycerol (4% in water) was added 0.5 mg of the lipopeptide from example 17 b). The mixture was heated to 80°C for 5 minutes and shaken. The solution was then cooled to ambient temperature and

the headspace was flushed with SF_6 gas. The vial was shaken on a Capmix (Espe Capmix) for 45 seconds and placed on a roller table for 5 min. The sample was centrifuged (Juan MR 14.11) at 2000 rpm for 5 minutes and the infranatant removed and replaced with destilled water. The headspace was again flushed with perfluorobutane and the sample was kept on a table roller until a homogenous appearance was obtained. The washing procedure was repeated.
The resulting ultrasound contrast agent was confirmed by Coulter counter, acoustic attenuation measurements and resistance to external pressure.

Example 26 - Preparation of PFB containing microbubbles of DSPG and endothelial cell binding lipopeptide for targeted ultrasound imaging.

To 0.8 ml of a solution containing DSPG (5mg/ ml) in propyleneglycol/glycerol (4% in water) was added 0.5 mg of the lipopeptide from example 17 b). The mixture was heated to 80°C for 5 minutes and shaken. The solution was then cooled to ambient temperature and the headspace was flushed with perfluorobutane. The vial was shaken on a Capmix (Espe Capmix) for 45 seconds and placed on a roller table for 5 min. The sample was centrifuged (Juan MR 14.11) at 2000 rpm for 5 minutes and the infranatant removed and replaced with destilled water. The headspace was again flushed with perfluorobutane and the sample was kept on a table roller until a homogenous appearance was obtained. The washing procedure was repeated.
The resulting ultrasound contrast agent was confirmed by Coulter counter, acoustic attenuation measurements and resistance to external pressure.
The microbubbles were tested in the in vitro assay as detailed in example 12. A gradual accumulation of bubbles binding to the cells was observed.

Example 27 - Preparation of PFP containing microbubbles of DSPG and endothelial cell binding lipopeptide for targeted ultrasound imaging.

To 0.8 ml of a solution containing DSPG (5mg/ ml) in propyleneglycol/glycerol (4% in water) was added 0.5 mg of the lipopeptide from example 17 b. The mixture was heated to 80°C for 5 minutes and shaken. The solution was then cooled to ambient temperature and the headspace was flushed with perfluoropropane. The vial was shaken on a Capmix (Espe Capmix) for 45 seconds and placed on a roller table for 5 min. The sample was centrifuged (Juan MR 14.11) at 2000 rpm for 5 minutes and the infranatant removed and replaced with destilled water. The headspace was again flushed with perfluorobutane and the sample was kept on a table roller until a homogenous appearance was obtained. The washing procedure was repeated. The resulting ultrasound contrast agent was confirmed by Coulter counter, acoustic attenuation measurements and resistance to external pressure.

The microbubbles were tested in the in vitro assay as detailed in example 12. A gradual accumulation of bubbles binding to the cells was observed.

Example 28 - Preparation of SF_6 containing microbubbles of DSPG and endothelial cell binding lipopeptide for targeted ultrasound imaging.

To 0.8 ml of a solution containing DSPG (5mg/ ml) in propyleneglycol/glycerol (4% in water) was added 0.5 mg of the lipopeptide from example 17 b). The mixture was heated to 80°C for 5 minutes and shaken. The solution was then cooled to ambient temperature and the headspace was flushed with SF_6 gas. The vial was shaken on a Capmix (Espe Capmix) for 45 seconds and placed on a roller table for 5 min. The sample was centrifuged (Juan MR 14.11) at 2000 rpm for 5 minutes and the infranatant

removed and replaced with destilled water. The headspace was again flushed with perfluorobutane and the sample was kept on a table roller until a homogenous appearance was obtained. The washing procedure was repeated. The resulting ultrasound contrast agent was confirmed by Coulter counter, acoustic attenuation measurements and resistance to external pressure.

Example 29 - Targeted gas-containing microbubbles of DSPS coated non-covalently with polylysine

DSPS (5 mg, Avanti) was weighed into a clean vial along with poly-L-lysine (Sigma, 0.2 mg). To the vial was added 1.0 ml of a solution of 1.4% propylene glycol/ 2.4% glycerol. The mixture was warmed to 80°C for 5 minutes. The sample was cooled to room temperature and the head space flushed with perfluorobutane gas. The vials were shaken in a cap mixer for 45 s and the microbubbles centrifuged at 1000 rpm for 3 minutes.

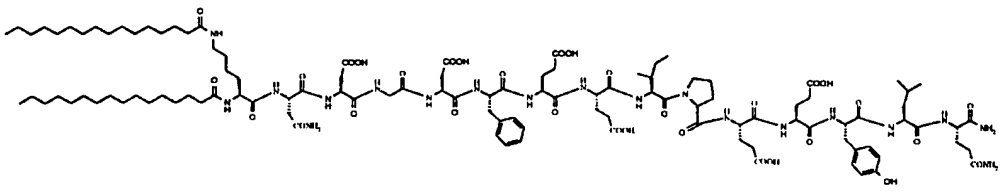
Following extensive washing with water, PBS and water the final solution was examined for polylysine content using MALDI MS. No polypeptide material was observed in the final wash solution.

Acetonitrile (0.5 ml) was then added and the microbubbles sonicated until all bubbles had burst. Analysis of the resulting solution for polylysine was again carried out using MALDI MS. The results were as follows:

	MALDI expected	MALDI found
Poly-L-lysine	786, 914, 1042, 1170 1177	790, 919, 1048,

Example 30 - Preparation of PFB gas-containing microbubbles of DSPS doped with a thrombus binding lipopeptide.

a) Synthesis of a lipopeptide with affinity for thrombi (Diplamitoyl-Lys-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln.NH_2).



The lipopeptide was synthesised on a ABI 433 A automatic peptide synthesiser starting with Rink amide resin (Novabiochem) on a 0.1 mmol scale using 1 mmol amino acid cartridges. All amino acids and palmitic acid were preactivated using HBTU before coupling.
The simultaneous removal of peptide from the resin and side-chain protecting groups was carried out in TFA containing 5% phenol, 5% EDT, 5% anisole and 5% H_2O for 2 h giving a crude product yield of 80 mg. Purification by preparative HPLC (Vydac 218TP1022 column) of a 20 mg aliquot of the crude material was carried out. After lyophilization 6 mg of pure material was obtained. The product was characterized by MALDI mass spectrometry and analytical HPLC.

b) Preparation of thromi-targeting ultrasound microbubbles.

DSPS (Avanti, 4.5 mg) and lipopeptide from a) (1.0 mg) were weighed into a vial and 0.8 ml of a solution of 1.4% propylene glycol/2.4% glycerol added. The mixture was warmed to 80°C for 5 minutes then the sample filtered through a 4 micron filter. After cooling to

room temperature the head space was flushed with perfluorobutane gas. The vials were shaken in a cap mixer for 45 s and the microbubbles washed extensively with deionised water. The bubbles were analysed by microscopy Coulter counter. MALDI-MS was used to confirm the presence of lipopeptide as previously described.

Example 31- Gas-filled microbubbles of DSPS comprising a lipopeptide consisting of a heparin sulphate binding peptide (KRKR) and a fibronectin peptide (WOPPRARI) for targeting and a lipopeptide containing atenolol for therapeutic applications

a) Synthesis of a protected atenolol derivative suitable for solid phase coupling.

i) Synthesis of methyl 4-[(2,3-epoxy)propoxy]phenylacetate

A mixture of methyl 4-hydroxyphenylacetate (4.98 g, 0.030 mol), epichlorohydrin (23.5 ml, 0.30 mol) and pyridine (121 μl, 1.5 mmol) was stirred at 85 °C for 2 h. The reaction mixture was cooled, and excess epichlorohydrin was distilled off (rotavapor). The residue was taken up in ethyl acetate, washed with brine and dried (Na_2SO_4). The solution was filtered and concentrated. The dark residue was chromatographed (silica, hexane/ethyl acetate 7:3) to give 2.25 g (34%) of a colourless oil. ^1H (300 MHz) and ^{13}C NMR (75 MHz) spectra were in accordance with the structure.

ii) Synthesis of methyl 4-[2-hydroxy-3-[(1-methylethyl)amino]propoxy]phenylaceta

te

A mixture of methyl 4-[(2,3-epoxy)propoxy]phenylacetate (2.00 g, 9.00 mmol), isopropylamine (23 ml, 0.27 mol) and water (1.35 ml, 74.7 mmol) was stirred at room temperature overnight. The reaction mixture was concentrated (rotavapor) and the oily residue was dissolved in chloroform and dried (Na_2SO_4). Filtration and concentration gave quantitative yield of a yellow oil that was used in the next step without further purification. The structure was verified by ^1H and ^{13}C NMR analysis.

iii) Synthesis of 4-[2-hydroxy-3-[(1-methylethyl)amino]propoxy]phenylaceti c acid hydrochloride

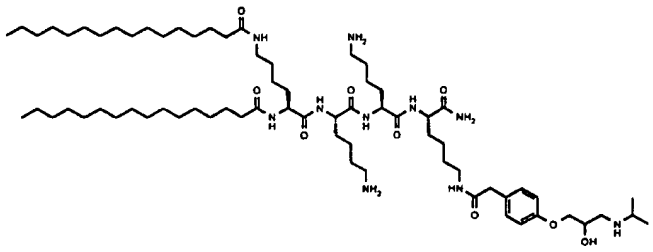
A solution of methyl 4-[2-hydroxy-3-[(1-methylethyl)amino]propoxy]phenylaceta te (563 mg, 2.00 mmol) in 6M hydrochloric acid (15 ml) was heated at 100 °C for 4h. The reaction mixture was concentrated (rotavapor) and the residue was taken up in water and lyophilised. ^1H and ^{13}C NMR spectra were in accordance with the strucure and MALDI mass spectrometry gave a M+H at 268 as expected.

iv) Synthesis of N-Boc-4-[2-hydroxy-3-[(1-methylethyl)amino]propoxy]phenylaceti c acid

A solution of the 4-[2-hydroxy-3-[(1-methylethyl)amino]propoxy]phenylaceti c acid hydrochloride (2.0 mmol) in water (2 ml) was added to a solution of sodium bicarbonate (0.60 g, 7.2 mmol) in water/dioxane (2:1, 15 ml). A solution of di-tert-butyl dicarbonate (0.48 g, 2.2 mmol) in dioxane (5 ml) was added. Progress of the reaction was monitored

by TLC analysis (silica, $CHCl_3$/MeOH/AcOH 85:10:5), and portions of di-tert-butyl dicarbonate were added until conversion was complete. The reaction mixture was poured onto water saturated with potassium hydrogensulphate and organic material was extracted into ethyl acetate. The organic phase was washed with water and brine, dried (Na_2SO_4) and filtered to give 0.6 g of crude material. The product was purified by chromatography (silica, $CHCl_3$/MeOH/AcOH 85:10:5). The solution was concentrated and the residue was taken up in glacial acetic acid and lyophilised. Yield 415 mg (56%), white solid. The structure was confirmed by ^1H and ^{13}C NMR analysis.

b) Synthesis of a lipopeptide functionalised with atenolol



The structure shown above was synthesised using a manual nitrogen bubbler apparatus starting with Fmoc protected Rink Amide MBHA resin (Novabiochem) on a 0.125 mmol scale, using amino acids from Novabiochem, palmitic acid from Fluka and the compound from a). Coupling was carried out using standard TBTU/HOBt/DIEA protocols. Simultaneous removal of the peptide from the resin and deprotection of side-chain protecting groups was carried out in TFA containing 5% EDT and 5% water for 2h. Crude material was precipitated from ether and purified by preparative liquid chromatography (Vydac 218TP1022 column) using a gradient of 70 to 100% B over 60 min (A = 0.1% TFA/water and B = 0.1% TFA/acetonitrile) at a flow rate of 10 ml/min. After lyophilisation a yield of 38 mg of pure material was obtained (analytical HPLC:

gradient 70-100% B over 20 min, A = 0.1% TFA/water and B = 0.1% TFA/acetonitrile, flow rate 1 ml/min, column Vydac 218TP54, detection UV 214 nm, retention time 25 min). Further characterisation was carried out using MALDI mass spectrometry (ACH matrix), giving M+H at 1258, expected 1257.

c) Preparation of gas-filled microbubbles of DSPS comprising a lipopeptide cosisting of a heparin sulphate binding peptide (KRKR) and a fibronectin peptide (WOPPRARI) and a lipopeptide containing atenolol for therapeutic applications

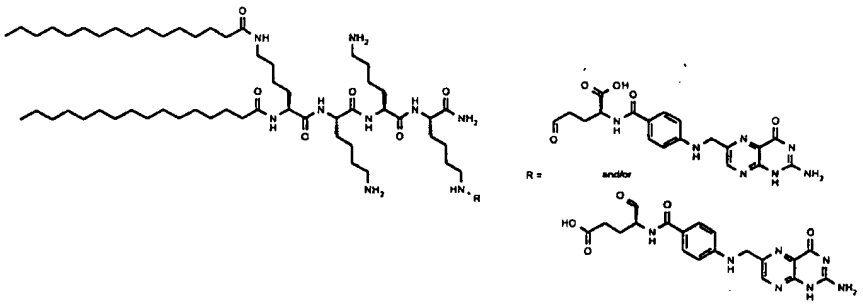
A solution of 1.4% propylene glycol / 2.4% glycerol (1.0 ml) was added to a mixture of DSPD (Avanti, 5.0 mg), product from Example 12 a) (0.5 mg) and product from b) (0.5 mg) in a vial. The mixture was sonicated for 5 min and then heated at 80 °C for 5 min (vial was shaken during warming). The solution was filtered and cooled. Head space was flushed with perfluorobutane gas and the vial was shaken in a cap mixer for 45s followed by extensive washing with deionised water.

Incorporation of atenolol containing lipopeptide into the bubbles was confirmed by MALDI-MS as follows. Ca 50 µl of microbubbles were transferred to a clean vial containing ca 100 µl of 90% methanol. The mixture was sonicated for 30s and analysed by MALDI-MS (ACH matrix), giving two M+H peaks at 2202 and 1259, corresponding to lipopeptide from Example 12 a) and to lipopeptide from b), respectively.

The microbubbles were tested in the in vitro assay as detailed in Example 12. A gradual accumulation of bubbles binding to the cells was observed.

Example 32 - Gas-filled microbubbles encapsulated with DSPS and a compound containing folic acid for diagnostic applications

a) Synthesis of a lipopeptide containing folic acid



The structure shown above, with was synthesised using a manual nitrogen bubbler apparatus starting with Fmoc protected Rink Amide MBHA resin (Novabiochem) on a 0.125 mmol scale, using amino acids from Novabiochem, palmitic acid from Fluka and folic acid from Acros. Coupling was carried out using standard TBTU/HOBt/DIEA protocols. Simultaneous removal of the peptide from the resin and deprotection of side-chain protecting groups was carried out in TFA containing 5% EDT and 5% water for 2h. Crude material was precipitated from ether and analysed by MALDI mass spectrometry, giving a M+H peak correspnding to the structure at 1435, expected 1430. The material was further characterised by analytical HPLC (column Vydac 218TP54, gradient 70-100% B over 20 min, A = 0.1% TFA/water and B = 0.1% TFA/acetonitrile, flow rate 1.0 ml/min), giving a product peak with retention time 27 min detected at UV 368 nm.

b) Preparation of gas-containing microbubbles of DSPS comprising a lipopeptide containing folic acid

A solution of 1.4% propylene glycol / 2.4% glycerol (1.0 ml) was added t a mixture of DSPS (Avanti, 4.5 mg) and

product from a) (0.5 mg) in a vial. Dilute ammonia (to pH 8) and DMSO (40 µl) were added and the mixture was sonicated for 5 min and then heated at 80 °C for 5 min (vial was shaken during warming). The solution was filtered and cooled. Head space was flushed with perfluorobutane gas and the vial was shaken in a cap mixer for 45s followed by extensive washing with deionised water.

Incorporation of structure from a) into the bubbles was confirmed by MALDI-MS as follows. Ca 50 µl of microbubbles were transferred to a clean vial containing ca 100 µl of 90% methanol. The mixture was sonicated for 30s and analysed by MALDI-MS (ACH matrix), giving a M+H peak at 1238 corresponding to structure from a).

The microbubbles were tested in the in vitro assay as detailed in example 12. A gradual accumulation of bubbles binding to the cells was observed.

Example 33 - Gas-filled microbubbles of phosphatidylserine comprising biotinamide-PEG-ß-Ala-Cholesterol and a cholesteryl ester of chlorambucil for diagnostic and therapeutic applications

a) Synthesis of cholesteryl N-Boc-ß-alaninate

DIC (510 µl) was added to a solution of Boc-ß-Ala-OH (1.25 g, 6.60 mmol) in dichloromethane (15 ml) under an inert atmosphere. The reaction mixture was stirred for 30 min and then transferred to a flask containing a solution of cholesterol (1.16 g, 3.00 mmol) and DMAP (367 mg, 3.00 mmol) in dichloromethane (15 ml). The reaction mixture was stirred for 2 h and then poured onto an aqeous solution of potassium hydrogensulphate. Phases were separated and the aq phase was extracted

with chloroform. Combined organic phases were washed with aq potassium hydrogensulphate and water and dried ($MgSO_4$) - FEK023/031-01. After filtration and evaporation the crude product was chromatographed (silica, chloroform/methanol 99:1) to give 1.63 g (97%) of white solid. The structure was confirmed by ^1H NMR (500 Mhz).

b) Synthesis of cholesteryl β-alaninate hydrochloride

A solution of compound from a) (279 mg, 0.500 mmol) in 1M hydrochloric acid in 1,4-dioxan (5 ml) was stirred at room temperature for 4h. The reaction mixture was concentrated to give a quantitative yield of cholesteryl β-alaninate hydrochloride. The structure was confirmed by 1H NMR (500 MHz) analysis and by MALDI mass spectrometry, giving a M+Na peak at 482, expected 481.

c) Biotin-PEG_{3400}-β-Ala-Cholesterol

To a solution of cholesteryl β-alaninate hydrochloride (15 mg, 0.03 mmol) in chloroform/wet methanol (2.6:1, 3 ml) was added triethylamine (42 μl, 0.30 mmol). The mixture was stirred for 10 minutes at room temperature and a solution of biotin-PEG3400-NHS (100 mg, 0.03 mmol) in 1,4-dioxane (1 ml) was added dropwise. After stirring at room temperature for 3 hours, the mixture was evapourated to dryness and the residue purified by flash chromatography to give white crystals, yield ; 102 mg (89%). The structure was verified by MALDI-MS and by NMR analysis.

d) Synthesis of cholesteryl 4-[4-[bis(2-chloroethyl)aminolphenyllbutanoate

DIC (170 μl, 1.10 mmol) was added to a solution of chlorambucil (Sigma, 669 mg, 2.20 mmol) in dry

dichloromethane (15 ml). The mixture was stirred at room temperature for 0.5 h and added to a solution of cholesterol (Aldrich, 387 mg, 1.00 mmol) and DMAP (122 mg, 1.00 mmol) in dichloromethane (10 ml). The reaction mixture was stirred overnight and then poured onto 5% sodium bicarbonate. The phases were separated and the organic phase was washed with brine and dried ($MgSO_4$). The solution was filtered and concentrated and the product was purified by column chromatography (silica, chloroform) to give 560 mg (83%) yield of colouless oil. The product was characterised by MALDI mass spectrometry, giving M+H at 674 as expected. Further characterisation was carried out using ^1H (500 MHz) and ^{13}C (125 MHz) NMR analysis, giving spectra in accordance with the structure.

e) Preparation of gas-filled microbubbles

A solution of 1.4% propylene glycol / 2.4% glycerol (1.0 ml) was added to a mixture of DSPS (Avanti, 5 mg) and product from c) (0.5 mg) and d) (0.5 mg) in a vial. The mixture was sonicated for 5 min and then heated at 80 °C for 5 min (vial was shaken during warming) and cooled. Head space was flushed with perfluorobutane gas and the vial was shaken in a cap mixer for 45s followed by extensive washing with deionised water. MALDI mass spectrometry showed no detectable level of compound from c and d) in the final wash solution.

Incorporation of compounds from c) and d) into the bubbles was confirmed by MALDI-MS as follows. Ca 50 µl of microbubbles were transferred to a clean vial containing ca 100 µl of 90% methanol. The mixture was sonicated for 30s and analysed by MALDI-MS (ACH-matrix), giving a M+H peaks corresponding to compounds from c) and d).

Example 34 -Flotation of endothelial cells by micro

bubbles with vectors that specifically bind to the endothelial cells

The human endothelial cell line ECV 304, derived from a normal umbilical cord (ATCC CRL-1998) was cultures in Nunc culture flasks (chutney 153732) in RPMI 1640 medium (Bio Whitaker) to which L-Glutamine 200 mM, Penicillin/Streptomycin (10.000 U/ml and 10.00 mcg/ml) and 10% Fetal Calf Serum (Hyclone Lot no AFE 5183) were added. The cells were subcultured following trypsination with a split ratio of 1:5 to 1:7 when reaching confluence.
2 mill. cells from trypsinated confluent cultures were added to each set of 5 centrifuge tubes. Then control microbubbles or bubbles carrying the vector including WQPPARI (example 12), or the endothelial cell binding peptide vector (example 14), were added at 2, 4, 6 ,8 or 10 mill bubbles per tube. The cells at the bottom of the tubes after centrifugation at 400 g for 5 minutes were counted with a Coulter counter. It was found the 4 or more microbubbles binding to a cell did bring the cells to top of the fluid in the centrifugation tube. All cells were floated by the endothelial cell binding peptide vector and about 50 % with the WQPPARI vector.

Example 35 - Gene transfer by PFB gas-filled microbubbles

This example is directed at the preparation of targeted microbubbles for gene transfer.

a) Preparation of DSPS lipopeptide bubbles/PFB gas, coated with polyl-L-lysine

DSPS (4,5 mg) and lipopeptide from 17 b) (0.5 mg) were weighed in two 2-ml vials. To each vial, 0.8 ml propyleneglycol/glycerol (4%) in water was added. The solution was heated at 80°C for 5 minutes and shaken. The

solution was then cooled to ambient temperature and the headspace flushed with perfluorobutane. The vials were shaken on a Capmix (Espe Capmix, 4450 oscillations/min) for 45 seconds and put on a roller table for 5 minutes. The content of the vials were mixed and the sample washed by centrifugation at 2000 rpm for 5 minutes. The infranatant was removed and the same volume of distilled water added. The washing procedure was repeated once.

Poly-L-lysine HBr (Sigma, 20.6 mg) was dissolved in 2 mL water then an aliquot (0.4 mL) made up to 2 mL water. To 1.2 mL of the diluted poly-L-lysine solution was added 0.12 mL of the DSPS-lipopeptide bubble suspension. Following incubation excess polylysine was removed by extensive washing with water.

b) Transfection of cells

Endothelial cells (ECV 304) were cultured in 6 well plates to a uniform subconfluent layer. A transfection mixture consisting of 5 µg DNA (an Enhanced Green Fluorescent Protein vector from CLONTECH) and 50 µl of microbubble suspension from a) in RPMI medium at a final volume of 250 µl was prepared. The mixture was left standing for 15 min at room temperature then 1 mL of complete RPMI medium added. The medium was removed from the cell culture dish, and the DNA-microbubble mixture added to the cells.
The cells were incubated in a cell culture incubator (37 °C).

c) Ultrasonic treatment

After 15 minutes incubation, selected wells were exposed to continious wave ultrasound of 1 MHz, 0.5 W/cm^2, for 30 seconds.

d) Incubation and examination

The cells were further incubated in the cell culture incubator (37 °C) for approximately 4 1/2 hours. The medium containing DNA-microbubbles was then removed by aspiration, and 2 ml complete RPMI medium was added. The cells were incubated for 40-70 hours before examination. Most of the medium was then removed, and the cells were examined by fluorescence microscopy. The results were compared to the results from control experiments were DNA or DNA-polylysine were added to the cells.

Claims

1. A targetable diagnostic and/or therapeutically active agent comprising a suspension in an aqueous carrier liquid of a reporter comprising gas-containing or gas-generating material, said reporter being coupled or linked to one or more vectors, characterised in that said vector or vectors are non-bioactive.

2. An agent as claimed in claim 1 wherein the gas comprises air, nitrogen, oxygen, carbon dioxide, hydrogen, an inert gas, a sulphur fluoride, selenium hexafluoride, a low molecular weight hydrocarbon, a ketone, an ester, a halogenated low molecular weight hydrocarbon or a mixture of any of the foregoing.

3. An agent as claimed in claim 2 wherein the gas comprises a perfluorinated ketone, perfluorinated ether or perfluorocarbon.

4. An agent as claimed in claim 2 wherein the gas comprises sulphur hexafluoride or a perfluoropropane, perfluorobutane or perfluoropentane.

5. An agent as claimed in any of the preceding claims comprising gas microbubbles stabilised by a coalescence-resistant surface membrane, a filmogenic protein, a polymer material, a non-polymeric and non-polymerisable wall-forming material or a surfactant.

6. An agent as claimed in claim 5 wherein said surfactant comprises at least one phospholipid.

7. An agent as claimed in claim 6 wherein at least 75% of the said surfactant material comprises phospholipid molecules individually bearing net overall charge.

8. An agent as claimed in claim 7 wherein at least 75% of the film-forming surfactant material comprises one or more phospholipids selected from phosphatidylserines, phosphatidylglycerols, phosphatidylinositols, phosphatidic acids and cardiolipins.

9. An agent as claimed in claim 8 wherein at least 80% of said phospholipids comprise phosphatidylserines.

10. An agent as claimed in any of the preceding claims comprising a combination of bioactive vectors, the biological activities of which are counterbalanced such that the combination is non-bioactive.

11. An agent as claimed in any of the preceding claims wherein the vector or vectors are monomeric or oligomeric.

12. An agent as claimed in any of claims 1 to 10 wherein the vector or vectors comprises non-bioactive peptides.

13. An agent as claimed in any of claims 1 to 10 wherein the vector or vectors are selected from antibodies; cell adhesion molecules; cell adhesion molecule receptors; cytokines; growth factors; peptide hormones and pieces thereof; non-bioactive binders of receptors for cell adhesion molecules; oligonucleotides and modified oligonucleotides; DNA-binding drugs; protease substrates/inhibitors; molecules generated from combinatorial libraries and proteins and peptides which bind to glucosaminoglycan side chains.

14. An agent as claimed in any of the preceding claims wherein the vector or vectors have affinity for targets at a level such that the agent interacts with but does not fixedly bind to said targets.

15. An agent as claimed in claim 14 wherein the vector or

vectors are selected from ligands for cell adhesion proteins and cell adhesion proteins which have corresponding ligands on endothelial cell surfaces.

16. An agent as claimed in any of the preceding claims wherein the vector or vectors are sited such that they are not readily exposed to the target.

17. An agent as claimed in any of the preceding claims wherein the vector is covalently or non-covalently coupled or linked to the reporter.

18. An agent as claimed in any one of claims 1 to 16 wherein the vector is coupled or linked to the reporter by means of electrostatic charge interaction.

19. An agent as claimed in any one of claims 1 to 16 wherein the vector is coupled or linked to the reporter by means of avidin-biotin and/or streptavidin-biotin interactions.

20. An agent as claimed in any of the preceding claims which further contains moieties which are radiocactive or are effective as X-ray contrast agents, light imaging probes or spin labels.

21. An agent as claimed in any of the preceding claims further comprising a therapeutic compound.

22. An agent as claimed in claim 21 wherein said therapeutic compound is an antineoplastic agent, blood product, biological response modifier, antifungal agent, hormone or hormone analogue, vitamin, enzyme, antiallergic agent, tissue factor inhibitor, platelet inhibitor, coagulation protein target inhibitor, fibrin formation inhibitor, fibrinolysis promoter, antiangiogenic, circulatory drug, metabolic potentiator, antitubercular,

antiviral, vasodilator, antibiotic, antiinflammatory, antiprotozoan, antirheumatic, narcotic, opiate, cardiac glycoside, neuromuscular blocker, sedative, local anaesthetic, general anaesthetic or genetic material.

23. An agent as claimed in claim 21 or claim 22 wherein said therapeutic compound is covalently coupled or linked to the reporter through disulphide groups.

24. An agent as claimed in claim 21 or claim 22 wherein a lipophilic or lipophilically-derivatised therapeutic compound is linked to the reporter through hydrophobic interactions.

25. A combined formulation comprising:

i) a first administrable composition comprising a pre-targeting vector having affinity for a selected target; and

ii) a second administrable composition comprising an agent as claimed in any of the preceding claims, said agent comprising a vector having affinity for said pre-targeting vector.

26. A combined formulation as claimed in claim 25 wherein said pre-targeting vector comprises a monoclonal antibody.

27. A combined formulation comprising:

i) a first administrable composition comprising an agent as claimed in any of claims 1 to 24, and

ii) a second administrable composition comprising a substance capable of displacing or releasing said agent from its target.

28. A combined formulation comprising:

i) a first administrable composition comprising an agent as claimed in claim 23, and

ii) a second administrable composition comprising a

reducing agent capable of reductively cleaving the disulphide groups coupling or linking the therapeutic compound and reporter in the agent of said first administrable composition.

29. A process for the preparation of a targetable diagnostic and/or therapeutically active agent as defined in claim 1 which comprises coupling or linking at least one non-bioactive vector to a reporter comprising gas-containing or gas-generating material.

30. A process as claimed in claim 29 wherein a therapeutic compound is also combined with the reporter.

31. Use of an agent as claimed in any of claims 1 to 24 as a targetable ultrasound contrast agent.

32. A method of generating enhanced images of a human or non-human animal body which comprises administering to said body an agent as claimed in any of claims 1 to 24 and generating an ultrasound, magnetic resonance, X-ray, radiographic or light image of at least a part of said body.

33. A method as claimed in claim 32 which comprises the steps:

i) administering to said body a pre-targeting vector having affinity for a selected target; and thereafter

ii) administering an agent as claimed in any of claims 1 to 24, said agent comprising a vector having affinity for said pre-targeting vector.

34. A method as claimed in claim 33 wherein said pre-targeting vector comprises a monoclonal antibody.

35. A method as claimed in claim 32 which comprises the

steps:

i) administering to said body an agent as claimed in any of claims 1 to 24; and thereafter

ii) administering a substance capable of displacing or releasing said agent from its target.

36. A method as claimed in any of claims 32 to 35 wherein said agent further comprises a therapeutic compound.

37. A method as claimed in claim 36 wherein said therapeutic compound is covalently coupled or linked to the reporter through disulphide groups, and a composition comprising a reducing agent capable of reductively cleaving said disulphide groups is subsequently administered.

38. A method for *in vitro* investigation of targeting by an agent as defined in any of claims 1 to 24 wherein cells expressing a target are fixedly positioned in a flow chamber, a suspension of said agent in a carrier liquid is passed through said chamber, and binding of said agent to said cells is examined.

39. A method as claimed in claim 38 wherein the flow rate of carrier liquid is controlled to simulate shear rates encountered *in vivo*.

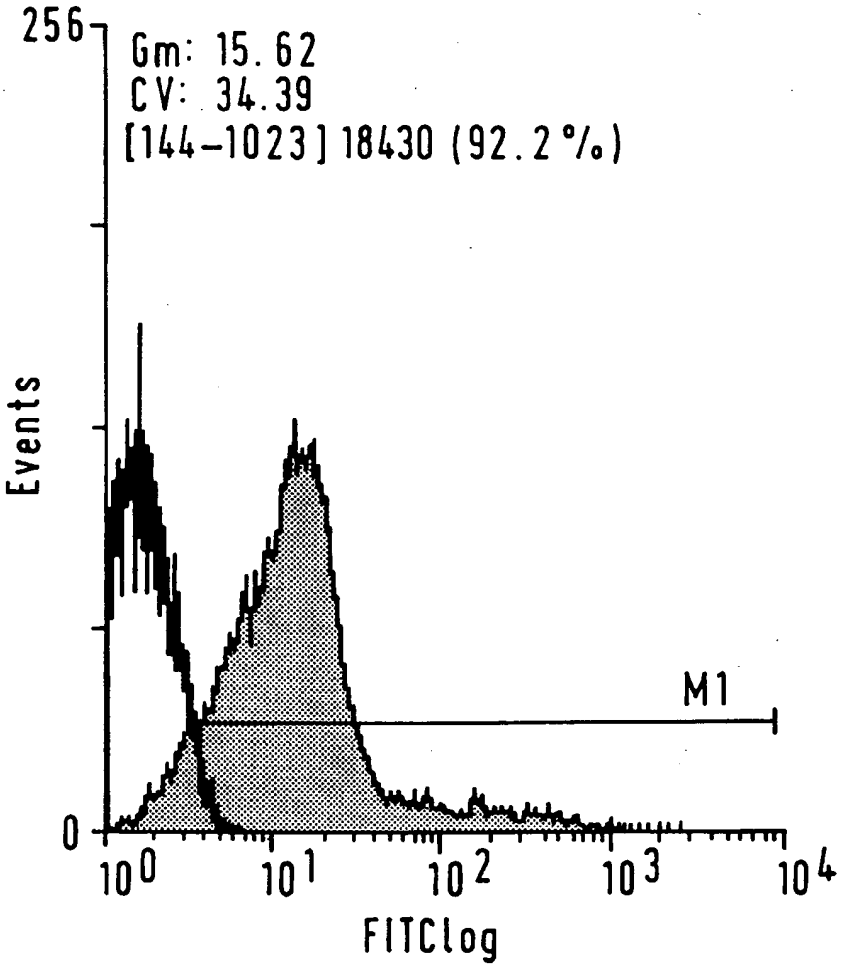

256
Gm: 15.62
CV: 34.39
[144–1023] 18430 (92.2 %)
Events
M1
0
10^0
10^1
10^2
10^3
10^4
FITClog

FIG. 1.

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(54) Title: A METHOD FOR ACTIVATING ONLY THE VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR-3 AND USES THEREOF

(57) Abstract: A method for activating only the vascular endothelial growth factor receptor-3 has been created. The method comprises administration of a composition comprising a polypeptide to an animal wherein the composition has the ability to stimulate one or more lymphatic vessel endothelial cells to proliferate, differentiate, migrate, or survive. Methods are also provided to selectively activate a VEGF receptor-3, to screen for neoplastic disease characterized by an increase in lymph vessel endothelial cells, and to identify lymph vessel endothelial cells.

A METHOD FOR ACTIVATING ONLY THE VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR-3 AND USES THEREOF

BACKGROUND OF THE INVENTION

The invention generally relates to a method for activating only the vascular endothelial growth factor receptor-3 (VEGFR-3) using mouse vascular endothelial growth factor-D (VEGF-D), and a method for promoting and maintaining lymphatic vascularization of tissue.

The two major components of the mammalian vascular system are the endothelial and smooth muscle cells. The endothelial cells form the lining of the inner surface of all blood vessels and lymphatic vessels in the mammal. The formation of new blood vessels can occur by two different processes, vasculogenesis or angiogenesis (for review *see* Risau, W., *Nature* **386:** 671-674, 1997). Vasculogenesis is characterized by the *in situ* differentiation of endothelial cell precursors to mature endothelial cells and association of these cells to form vessels, such as occurs in the formation of the primary vascular plexus in the early embryo. In contrast, angiogenesis, the formation of blood vessels by growth and branching of pre-existing vessels, is important in later embryogenesis and is responsible for the blood vessel growth which occurs in the adult. Angiogenesis is a physiologically complex process involving proliferation of endothelial cells, degradation of extracellular matrix, branching of vessels and subsequent cell adhesion events. In the adult, angiogenesis is tightly controlled and limited under normal circumstances to the female reproductive system. However angiogenesis can be switched on in response to tissue damage. Importantly solid tumors are able to induce angiogenesis in surrounding tissue, thus sustaining tumor growth and facilitating the formation of metastases (Folkman,

J., *Nature Med.* **1**: 27-31, 1995). The molecular mechanisms underlying the complex angiogenic processes are far from being understood.

Lymphatic vessels are very different in structure from arteriae, veins and capillaries. Lymph vessels are characterized by an extremely permeable, thin endothelial lining devoid of a basal lamina. Simple end-to-end cell junctions and interdigitating and especially junctions between endothelial cells are a characteristic feature of lymphatic vessels. In addition, the lymphatics typically lack supporting cells, such as pericytes and smooth muscle cells (Leak, L., *Microvasc Res* **2**: 361-391, 1970; Leak, L., *J Cell Biol.*, **50**: 300-323, 1971; and Leak and Jamuar, *Am Rev Respir Dis.* **128**: S59-S65, 1983). The differences in the structure and cellular composition of lymphatic and non-lymphatic vessels suggest that the lymphatic endothelial cells may represent a very differentiated form of endothelial cells (Taiplae, J. *et al.*, *Cur. Topics Micro. Immunol.* **237**: 85-96, 1999).

A major function of the lymphatic system is to provide fluid return from tissues and to transport many extravascular substances back to the blood. In addition, during the process of maturation, lymphocytes leave the blood, migrate through lymphoid organs and other tissues, and enter the lymphatic vessels, and return to the blood through the thoracic duct. Specialized venules, high endothelial venules (HEVs), bind lymphocytes again and cause their extravasation into tissues. The lymphatic vessels, and especially the lymph nodes, thus play an important role in immunology and in the development of metastasis of different tumors. Unlike blood vessels, the embryonic origin of the lymphatic system is not as clear and at least three different theories exist as to its origin. Lymphatic vessels are difficult to

identify due to the absence of known specific markers available for them.

Lymphatic vessels are most commonly studied with the aid of lymphography. In lymphography, X-ray contrast medium is injected directly into a lymphatic vessel. The contrast medium gets distributed along the efferent drainage vessels of the lymphatic system and is collected in the lymph nodes. The contrast medium can stay for up to half a year in the lymph nodes, during which time X-ray analyses allow the follow-up of lymph node size and architecture. This diagnostic is especially important in cancer patients with metastases in the lymph nodes and in lymphatic malignancies, such as lymphoma. However, improved materials and methods for imaging lymphatic tissues are needed in the art.

Angiogenesis is also involved in a number of pathologic conditions, where it plays a role or is involved directly in different sequelae of the disease. Some examples include neovascularization associated with various liver diseases, neovascular sequelae of diabetes, neovascular sequelae to hypertension, neovascularization in post-trauma, neovascularization due to head trauma, neovascularization in chronic liver infection (e.g. chronic hepatitis), neovascularization due to heat or cold trauma, dysfunction related to excess of hormone, creation of hemangiomas and restenosis following angioplasty.

Because of the crucial role of angiogenesis in so many physiological and pathological processes, factors involved in the control of angiogenesis have been intensively investigated. A number of growth factors have been shown to be involved in the regulation of angiogenesis; these include fibroblast growth factors (FGFs), platelet-derived growth factor (PDGF), transforming growth factor alpha (TGFα), and

hepatocyte growth factor (HGF). See for example Folkman *et al.*, *J. Biol. Chem.*, **267:** 10931-10934, 1992 for a review.

It has been suggested that a particular family of endothelial cell-specific growth factors, the vascular endothelial growth factors (VEGFs), and their corresponding receptors is primarily responsible for stimulation of endothelial cell growth and differentiation, and for certain functions of the differentiated cells. These factors are members of the PDGF/VEGF family, and appear to act primarily via endothelial receptor tyrosine kinases (RTKs). The PDGF/VEGF family of growth factors belongs to the cystine-knot superfamily of growth factors, which also includes the neurotrophins and transforming growth factor-ß.

Eight different proteins have been identified in the PDGF/VEGF family, namely two PDGFs (A and B), VEGF and five members that are closely related to VEGF. The five members closely related to VEGF are: VEGF-B, described in International Patent Application PCT/US96/02957 (WO 96/26736) and in U.S. Patents 5,840,693 and 5,607,918 by Ludwig Institute for Cancer Research and The University of Helsinki; VEGF-C or VEGF2, described in Joukov *et al.*, *EMBO J.*, **15:** 290-298, 1996, Lee *et al.*, *Proc. Natl. Acad. Sci. USA*, **93:** 1988-1992, 1996, and U.S. Patents 5,932,540 and 5,935,540 by Human Genome Sciences, Inc; VEGF-D, described in International Patent Application No. PCT/US97/14696 (WO 98/07832), and Achen *et al.*, *Proc. Natl. Acad. Sci. USA*, **95:** 548-553, 1998; the placenta growth factor (PlGF), described in Maglione *et al.*, Proc. Natl. Acad. Sci. USA, **88:** 9267-9271, 1991; and VEGF3, described in International Patent Application No. PCT/US95/07283 (WO 96/39421) by Human Genome Sciences, Inc. Each VEGF family member has between 30% and 45% amino acid sequence identity with VEGF. The VEGF family members share a VEGF homology domain which contains the six

cysteine residues which form the cystine-knot motif. Functional characteristics of the VEGF family include varying degrees of mitogenicity for endothelial cells, induction of vascular permeability and angiogenic and lymphangiogenic properties.

Vascular endothelial growth factor (VEGF) is a homodimeric glycoprotein that has been isolated from several sources. Alterative mRNA splicing of a single VEGF gene gives rise to five isoforms of VEGF. VEGF shows highly specific mitogenic activity for endothelial cells. VEGF has important regulatory functions in the formation of new blood vessels during embryonic vasculogenesis and in angiogenesis during adult life (Carmeliet *et al.*, *Nature*, **380:** 435-439, 1996; Ferrara *et al.*, *Nature*, **380:** 439-442, 1996; reviewed in Ferrara and Davis-Smyth, *Endocrine Rev.*, **18:** 4-25, 1997). The significance of the role played by VEGF has been demonstrated in studies showing that inactivation of a single VEGF allele results in embryonic lethality due to failed development of the vasculature (Carmeliet *et al.*, *Nature*, **380:** 435-439, 1996; Ferrara *et al.*, *Nature*, **380:** 439-442, 1996). The isolation and properties of VEGF have been reviewed; see Ferrara *et al.*, *J. Cellular Biochem.*, **47:** 211-218, 1991 and Connolly, *J. Cellular Biochem.*, **47:** 219-223, 1991.

In addition VEGF has strong chemoattractant activity towards monocytes, can induce the plasminogen activator and the plasminogen activator inhibitor in endothelial cells, and can also induce microvascular permeability. Because of the latter activity, it is sometimes referred to as vascular permeability factor (VPF). VEGF is also chemotactic for certain hematopoetic cells. Recent literature indicates that VEGF blocks maturation of dendritic cells and thereby reduces the effectiveness of the immune response to tumors

(many tumors secrete VEGF) (Gabrilovich *et al.*, *Blood* **92**: 4150-4166, 1998; Gabrilovich *et al.*, *Clinical Cancer Research* **5**: 2963-2970, 1999).

VEGF-B has similar angiogenic and other properties to those of VEGF, but is distributed and expressed in tissues differently from VEGF. In particular, VEGF-B is very strongly expressed in heart, and only weakly in lung, whereas the reverse is the case for VEGF. This suggests that VEGF and VEGF-B, despite the fact that they are co-expressed in many tissues, may have functional differences.

VEGF-B was isolated using a yeast co-hybrid interaction trap screening technique by screening for cellular proteins which might interact with cellular retinoic acid-binding protein type I (CRABP-I). Its isolation and characteristics are described in detail in PCT/US96/02957 and in Olofsson *et al.*, *Proc. Natl. Acad. Sci. USA*, **93**: 2576-2581, 1996.

VEGF-C was isolated from conditioned media of the PC-3 prostate adenocarcinoma cell line (CRL1435) by screening for ability of the medium to produce tyrosine phosphorylation of the endothelial cell-specific receptor tyrosine kinase VEGFR-3 (Flt4), using cells transfected to express VEGFR-3. VEGF-C was purified using affinity chromatography with recombinant VEGFR-3, and was cloned from a PC-3 cDNA library. Its isolation and characteristics are described in detail in Joukov *et al.*, *EMBO J.*, **15**: 290-298, 1996.

VEGF-D was isolated from a human breast cDNA library, commercially available from Clontech, by screening with an expressed sequence tag obtained from a human cDNA library designated "Soares Breast 3NbHBst" as a hybridization probe (Achen *et al.*, *Proc. Natl. Acad. Sci. USA*, **95**: 548-553, 1998). Its isolation and characteristics are described in detail in International Patent Application No. PCT/US97/14696 (WO98/07832).

In PCT/US97/14696, the isolation of a biologically active fragment of VEGF-D, designated VEGF-DΔNΔC, is also described. This fragment consists of VEGF-D amino acid residues 93 to 201 linked to the affinity tag peptide FLAG®. The entire disclosure of the International Patent Application PCT/US97/14696 (WO 98/07832) is incorporated herein by reference.

The VEGF-D gene is broadly expressed in the adult human, but is certainly not ubiquitously expressed. VEGF-D is strongly expressed in heart, lung and skeletal muscle. Intermediate levels of VEGF-D are expressed in spleen, ovary, small intestine and colon, and a lower expression occurs in kidney, pancreas, thymus, prostate and testis. No VEGF-D mRNA was detected in RNA from brain, placenta, liver or peripheral blood leukocytes.

PlGF was isolated from a term placenta cDNA library. Its isolation and characteristics are described in detail in Maglione *et al.*, *Proc. Natl. Acad. Sci. USA*, **88:** 9267-9271, 1991. Presently its biological function is not well understood.

VEGF3 was isolated from a cDNA library derived from colon tissue. VEGF3 is stated to have about 36% identity and 66% similarity to VEGF. The method of isolation of the gene encoding VEGF3 is unclear and no characterization of the biological activity is disclosed.

Similarity between two proteins is determined by comparing the amino acid sequence and conserved amino acid substitutions of one of the proteins to the sequence of the second protein, whereas identity is determined without including the conserved amino acid substitutions.

As noted above, the PDGF/VEGF family members act primarily by binding to receptor tyrosine kinases. In general, receptor tyrosine kinases are glycoproteins, which

consist of an extracellular domain capable of binding a specific growth factor(s), a transmembrane domain, which is usually an alpha-helical portion of the protein, a juxtamembrane domain, which is where the receptor may be regulated by, e.g., protein phosphorylation, a tyrosine kinase domain, which is the enzymatic component of the receptor and a carboxy-terminal tail, which in many receptors is involved in recognition and binding of the substrates for the tyrosine kinase.

Five endothelial cell-specific receptor tyrosine kinases have been identified, belonging to two distinct subclasses: three vascular endothelial cell growth factor receptors, VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1), VEGFR-3 (Flt4), and the two receptors of the Tie family, Tie and Tie-2 (Tek). These receptors differ in their specificity and affinity. All of these have the intrinsic tyrosine kinase activity which is necessary for signal transduction. The VEGFRs are subclass-III receptor tyrosine kinases, homologous to the platelet-derived growth factor-receptor family, having seven immunoglobulin homology domains in the extracellular domain and a tyrosine kinase intracellular domain split by kinase insert sequence.

The only receptor tyrosine kinases known to bind VEGFs are VEGFR-1, VEGFR-2 and VEGFR-3. VEGFR-1 and VEGFR-2 bind VEGF with high affinity, and VEGFR-1 also binds VEGF-B and PlGF. VEGF-C has been shown to be the ligand for VEGFR-3, and it also activates VEGFR-2 (Joukov *et al.*, *The EMBO Journal*, **15:** 290-298, 1996). VEGF-D binds to both VEGFR-2 and VEGFR-3 (Achen *et al.*, *Proc. Natl. Acad. Sci. USA*, **95:** 548-553, 1998). A ligand for Tie-2 (Tek) has been described in International Patent Application No. PCT/US95/12935 (WO 96/11269) by Regeneron Pharmaceuticals, Inc. The ligand for Tie has not yet been identified.

Recently, a novel 130-135 kDa VEGF isoform specific receptor has been purified and cloned (Soker *et al.*, *Cell*, **92:** 735-745, 1998). The VEGF receptor was found to specifically bind the $VEGF_{165}$ isoform via the exon 7 encoded sequence, which shows weak affinity for heparin (Soker *et al.*, *Cell*, **92:** 735-745, 1998). Surprisingly, the receptor was shown to be identical to human neuropilin-1 (NP-1), a receptor involved in early stage neuromorphogenesis. PlGF-2 also appears to interact with NP-1 (Migdal *et al.*, *J. Biol. Chem.*, **273:** 22272-22278, 1998).

VEGFR-1, VEGFR-2 and VEGFR-3 are expressed differently by endothelial cells. Generally, both VEGFR-1 and VEGFR-2 are expressed in blood vessel endothelia (Oelrichs *et al.*, *Oncogene*, **8:** 11-18, 1992; Kaipainen *et al.*, *J. Exp. Med.*, **178:** 2077-2088, 1993; Dumont *et al.*, *Dev. Dyn.*, **203:** 80-92, 1995; Fong *et al.*, *Dev. Dyn.*, **207:** 1-10, 1996) and VEGFR-3 is mostly expressed in the lymphatic endothelium of adult tissues (Kaipainen *et al.*, *Proc. Natl. Acad. Sci. USA*, **9:** 3566-3570, 1995). VEGFR-3 is also expressed in the blood vasculature surrounding tumors.

Although VEGFR-1 is mainly expressed in endothelial cells during development, it can also be found in hematopoetic precursor cells during early stages of embryogenesis (Fong *et al.*, *Nature*, **376:** 66-70, 1995). In adults, monocytes and macrophages also express this receptor (Barleon *et al.*, *Blood*, **87:** 3336-3343, 1995). In embryos, VEGFR-1 is expressed by most, if not all, vessels (Breier *et al.*, *Dev. Dyn.*, **204:** 228-239, 1995; Fong *et al.*, *Dev. Dyn.*, **207:** 1-10, 1996).

The receptor VEGFR-3 is widely expressed on endothelial cells during early embryonic development but as embryogenesis proceeds becomes restricted to venous endothelium and then to the lymphatic endothelium (Kaipainen *et al.*, *Cancer Res.*,

54: 6571-6577, 1994; Kaipainen *et al.*, *Proc. Natl. Acad. Sci. USA*, **92:** 3566-3570, 1995). VEGFR-3 is expressed on lymphatic endothelial cells in adult tissues. This receptor is essential for vascular development during embryogenesis.

The essential, specific role in vasculogenesis, angiogenesis and/or lymphangiogenesis of VEGFR-1, VEGFR-2, VEGFR-3, Tie and Tie-2 has been demonstrated by targeted mutations inactivating these receptors in mouse embryos. Disruption of the VEGFR genes results in aberrant development of the vasculature leading to embryonic lethality around midgestation. Analysis of embryos carrying a completely inactivated VEGFR-1 gene suggests that this receptor is required for functional organization of the endothelium (Fong *et al.*, *Nature*, **376:** 66-70, 1995). However, deletion of the intracellular tyrosine kinase domain of VEGFR-1 generates viable mice with a normal vasculature (Hiratsuka *et al.*, *Proc. Natl. Acad. Sci. USA*, **95:** 9349-9354, 1998). The reasons underlying these differences remain to be explained but suggest that receptor signalling via the tyrosine kinase is not required for the proper function of VEGFR-1. Analysis of homozygous mice with inactivated alleles of VEGFR-2 suggests that this receptor is required for endothelial cell proliferation, hematopoesis and vasculogenesis (Shalaby *et al.*, *Nature*, **376:** 62-66, 1995; Shalaby *et al.*, *Cell*, **89:** 981-990, 1997). Targeted inactivation of both copies of the VEGFR-3 gene in mice resulted in defective blood vessel formation characterized by abnormally organized large vessels with defective lumens, leading to fluid accumulation in the pericardial cavity and cardiovascular failure at post-coital day 9.5 (Dumont *et al.*, *Science*, **282:** 946-949, 1998). On the basis of these findings it has been proposed that VEGFR-3 is required for the maturation of primary vascular networks into larger blood vessels. However, the role of VEGFR-3 in the

development of the lymphatic vasculature could not be studied in these mice because the embryos died before the lymphatic system emerged. Nevertheless it is assumed that VEGFR-3 plays a role in development of the lymphatic vasculature and lymphangiogenesis given its specific expression in lymphatic endothelial cells during embryogenesis and adult life. This is supported by the finding that ectopic expression of VEGF-C, a ligand for VEGFR-3, in the skin of transgenic mice, resulted in lymphatic endothelial cell proliferation and vessel enlargement in the dermis. Furthermore this suggests that VEGF-C may have a primary function in lymphatic endothelium, and a secondary function in angiogenesis and permeability regulation which is shared with VEGF (Joukov *et al.*, *EMBO J.*, **15**: 290-298, 1996).

Homologs of the human VEGF receptor-3 have been cloned from the mouse and quail (Finnerty, *Oncogene*, **8**: 2293-2298, 1993; and Eichmann *et al.*, *Gene*, **174**: 3-8, 1996). They are relatively conserved in evolution, the quail having 70% amino acid identity with the human receptor and having similar ligand receptor binding.

There is tremendous interest in the development of pharmacological agents which could antagonize the receptor-mediated actions of VEGFs (Martiny-Baron and Marme, *Curr. Opin. Biotechnol.* **6**: 675-680, 1995). Monoclonal antibodies to VEGF have been shown to suppress tumor growth *in vivo* by inhibiting tumor-associated angiogenesis (Kim *et al.*, *Nature* **362**: 841-844, 1993). Similar inhibitory effects on tumor growth have been observed in model systems resulting from expression of either antisense RNA for VEGF (Saleh *et al.*, *Cancer Res.* **56**: 393-401, 1996) or a dominant-negative VEGFR-2 mutant (Millauer *et al.*, *Nature* **367**: 576-579, 1994).

However, tumor inhibition studies with neutralizing antibodies suggested that other angiogenic factors besides

VEGF may be involved (Kim, K. *et al.*, *Nature* **362**: 841-844, 1993). Furthermore, the activity of angiogenic factors other than VEGF in malignant melanoma is suggested by the finding that not all melanomas express VEGF (Issa, R. *et al.*, *Lab Invest* **79**: 417-425, 1999).

The biological functions of the different members of the VEGF family are currently being elucidated. Of particular interest are the properties of VEGF-D and VEGF-C. These proteins bind to both VEGFR-2 and VEGFR-3, localized on vascular and lymphatic endothelial cells, respectively. They are also closely related in primary structure (48% amino acid identity). Both factors are mitogenic for endothelial cells *in vitro*. Recently, VEGF-C was shown to be angiogenic in the mouse cornea model and in the avian chorioallantoic membrane (Cao *et al.*, *Proc. Natl. Acad. Sci. USA* **95**: 14389-14394, 1998) and was able to induce angiogenesis in the setting of tissue ischemia (Witzenbichler *et al.*, *Am. J. Pathol.* **153**: 381-394, 1998). Furthermore, VEGF-C stimulated lymphangiogenesis in the avian chorioallantoic membrane (Oh *et al.*, *Dev. Biol.* **188**: 96-109, 1997) and in a transgenic mouse model (Jeltsch *et al.*, *Science* **276**: 1423-1425, 1997). VEGF-D was shown to be angiogenic in the rabbit cornea (Marconcini *et al.*, *Proc. Natl. Acad. Sci. USA* **96**: 9671-9676, 1999). The lymphangiogenic capacity of VEGF-D has not yet been reported, however, given that VEGF-D, like VEGF-C, binds and activates VEGFR-3, a receptor thought to signal for lymphangiogenesis (Taipale *et al.*, *Cur. Topics Micro. Immunol.* **237**: 85-96, 1999), it is highly likely that VEGF-D is lymphangiogenic. VEGF-D and VEGF-C may be of particular importance for the malignancy of tumors, as metastases can spread via either blood vessels or lymphatic vessels; therefore molecules which stimulate angiogenesis or lymphangiogenesis could contribute toward malignancy. This

has already been shown to be the case for VEGF. It is noteworthy that VEGF-D gene expression is induced by c-Fos, a transcription factor known to be important for malignancy (Orlandini *et al.*, *Proc. Natl. Acad. Sci. USA* **93**: 11675-11680, 1996). It is speculated that the mechanism by which c-Fos contributes to malignancy is the upregulation of genes encoding angiogenic factors. Tumor cells deficient in c-fos fail to undergo malignant progression, possibly due to an inability to induce angiogenesis (Saez, E. *et al.*, *Cell* **82**: 721-732, 1995). This indicates the existence of an angiogenic factor up-regulated by c-fos during tumor progression.

As shown in Figure 1, the predominant intracellular form of human VEGF-D is a homodimeric propeptide that consists of the VEGF/PDGF Homology Domain (VHD) and the N- and C-terminal propeptides. After secretion, this polypeptide is proteolytically cleaved (Stacker, S.A. *et al.*, *J Biol Chem* **274**: 32127-32136, 1999). Proteolytic processing (at positions marked by black arrowheads) gives rise to partially processed forms and a fully processed, mature form which consists of dimers of the VHD. With human VEGF-D, the VHD consists of residues 93 to 201 of full length VEGF-D and contains the binding sites for both VEGFR-2 and VEGFR-3. The mature form binds both VEGFR-2 and VEGFR-3 with much higher affinity than the unprocessed form (Stacker, S.A. *et al.*, *J Biol Chem* **274**: 32127-32136, 1999).

The description of the cloning of the mouse homolog of VEGF-D is also found in International Patent Application PCT/US97/14696 (WO 98/07832). With the mouse, it was found that there are two isoforms. The longer amino acid sequence is designated mVEGF-D1, and the shorter sequence is designated mVEGF-D2. The nucleotide sequences of the cDNAs encoding mVEGF-D1 and mVEGF-D2 are found in SEQ ID NOs:1 and

3, respectively. The deduced amino acid sequences for mVEGF-D1 and mVEGF-D2 are found in SEQ ID NOs:2 and 4, respectively. The differences between the amino acid sequences are:

i) an insertion of five amino acids (DFSFE)(SEQ ID NO:5) after residue 30 in mVEGF-D1 in comparison to mVEGF-D2;

ii) complete divergence of the C-terminal ends after residue 317 in mVEGF-D1 and residue 312 in mVEGF-D2, which results in mVEGF-D1 being considerably longer.

VEGF-D is highly conserved between mouse and man. 85% of the amino acid residues of human VEGF-D are identical in mouse VEGF-D1. It is also predicted that the predominant intracellular form of mouse VEGF-D is a homodimeric propeptide that consists of the VEGF/PDGF Homology Domain (VHD) and the N- and C-terminal propeptides. The mouse VHD consists of residues 92 to 201 (SEQ ID NO:6) of the full length mouse VEGF-D2 (SEQ ID NO:4).

SUMMARY OF THE INVENTION

The invention generally relates to a method for promoting and/or maintaining lymphatic vascularization of tissue that involves activation of only the vascular endothelial growth factor receptor-3 (VEGFR-3) using mouse vascular endothelial growth factor-D (VEGF-D).

According to a first aspect, the invention provides a method for stimulating proliferation and/or maintaining of only lymph vessel endothelial cells. The method comprises administering to the endothelial cells an effective amount of a composition comprising a polypeptide with the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or a fragment thereof. Preferably the polypeptide has at least

90%, and more preferably at least 95% identity to the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or a fragment thereof.

As herein used the term "fragment thereof" refers to fragments of a polypeptide having at least a 90% sequence identity to the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 and that has the ability to only stimulate one or more of lymphatic vessel endothelial cells to proliferate, differentiate, migrate or survive.

According to a second aspect, the invention provides a method for activation of only the VEGF receptor-3 which comprises administering to a cell bearing this receptor an effective amount of a composition comprising a polypeptide having the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or a fragment thereof. Preferably the polypeptide has at least 90%, and more preferably at least 95% identity to the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or a fragment thereof. This method can be carried out *in vivo* or *in vitro*.

Since the polypeptide specifically activates the VEGF receptor-3, this polypeptide can be used to stimulate endothelial cell proliferation in a situation where VEGF receptor-2 is not activated. Accordingly, the invention provides for a method for specific activation of VEGF receptor-3 and not the VEGF receptor-2.

In addition, variant forms of the mouse VEGF-D polypeptide which result from naturally-occurring allelic variants of the nucleic acid sequence encoding mouse VEGF-D are encompassed within the scope of the invention. Allelic variants are well known in the art, and represent alternative forms or a nucleic acid sequence which comprise substitution, deletion or addition of one or more nucleotides, but which do

not result in any substantial functional alteration of the encoded polypeptide.

Where amino acid substitution is used to create a variant, the substitution is conservative, i.e. an amino acid is replaced by one of similar size and with similar charge properties.

As used herein, the term "conservative substitution" denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative substitutions include the substitution of one hydrophobic residue such as isoleucine, valine, leucine, alanine, cysteine, glycine, phenylalanine, proline, tryptophan, tyrosine, norleucine or methionine for another, or the substitution of one polar residue for another, such as the substitution of arginine for lysine, glutamic acid for aspartic acid, or glutamine for asparagine, and the like. Neutral hydrophilic amino acids which can be substituted for one another include asparagine, glutamine, serine and threonine. The term "conservative substitution" also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid.

As such, it should be understood that in the context of the present invention, a conservative substitution is recognized in the art as a substitution of one amino acid for another amino acid that has similar properties. Exemplary conservative substitutions are set out in the following Table A from WO 97/09433.

Table A

Conservative Substitutions I

SIDE CHAIN CHARACTERISTIC	AMINO ACID
Aliphatic	
Non-polar	G A P
	I L V
Polar - uncharged	C S T M
	N Q
Polar - charged	D E
	K R
Aromatic	H F W Y
Other	N Q D E

Alternatively, conservative amino acids can be grouped as described in Lehninger, [Biochemistry, Second Edition; Worth Publishers, Inc. NY:NY (1975), pp.71-77] as set out in the following Table B.

Table B

Conservative Substitutions II

SIDE CHAIN CHARACTERISTIC	AMINO ACID
Non-polar (hydrophobic)	
A. Aliphatic:	A L I V P
B. Aromatic:	F W
C. Sulfur-containing:	M
D. Borderline:	G
Uncharged-polar	
A. Hydroxyl:	S T Y
B. Amides:	N Q
C. Sulfhydryl:	C
D. Borderline:	G
Positively Charged (Basic):	K R H
Negatively Charged (Acidic):	D E

Exemplary conservative substitutions are set out in the following Table C.

Table C

Conservative Substitutions III

Original Residue	Exemplary Substitution
Ala (A)	Val, Leu, Ile
Arg (R)	Lys, Gln, Asn
Asn (N)	Gln, His, Lys, Arg
Asp (D)	Glu
Cys (C)	Ser
Gln (Q)	Asn
Glu (E)	Asp
His (H)	Asn, Gln, Lys, Arg
Ile (I)	Leu, Val, Met, Ala, Phe,
Leu (L)	Ile, Val, Met, Ala, Phe
Lys (K)	Arg, Gln, Asn
Met (M)	Leu, Phe, Ile
Phe (F)	Leu, Val, Ile, Ala
Pro (P)	Gly
Ser (S)	Thr
Thr (T)	Ser
Trp (W)	Tyr, Phe
Tyr (Y)	Trp, Phe, Thr, Ser
Val (V)	Ile, Leu, Met, Phe, Ala

As used herein, the term "mouse VEGF-D" collectively refers to the polypeptide having the amino acid of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 and fragments thereof and other variants which have the biological activity of mouse VEGF-D as herein defined. Those skilled in the art will recognize that there is considerable latitude in amino acid sequence charges which can occur naturally or be engineered without affecting biological activity of the polypeptide. It is preferred that the variant polypeptides be at least 90% and more preferably be at least 95% identical to the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 and fragments thereof. Percent sequence identity is determined by conventional methods. See, for example, Altschul *et al*, Bull. Math. Bio., 1986 48 603-616 and Henikoff and Henikoff, Proc. Natl. Acad. Sci. USA, 1992 89 10915-10919.

The polypeptide having at least a 90% identity with the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or a fragment thereof may be employed in combination with a suitable pharmaceutical carrier. The resulting compositions comprise a therapeutically effective amount of the claimed polypeptide, and a pharmaceutically acceptable non-toxic salt thereof, and a pharmaceutically acceptable solid or liquid carrier or adjuvant. Examples of such a carrier or adjuvant include, but are not limited to, saline, buffered saline, Ringer's solution, mineral oil, talc, corn starch, gelatin, lactose, sucrose, microcrystalline cellulose, kaolin, mannitol, dicalcium phosphate, sodium chloride, alginic acid, dextrose, water, glycerol, ethanol, thickeners, stabilizers, suspending agents and combinations thereof. Such compositions may be in the form of solutions, suspensions, tablets, capsules, creams, salves, elixirs, syrups, wafers, ointments or other conventional forms. The

formulation to suit the mode of administration. Compositions comprising a polypeptide having at least a 90% identity with the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or a fragment thereof will contain from about 0.1% to 90% by weight of the active compound, and most generally from about 10% to 30%.

For intramuscular preparations, a sterile formulation, preferably a suitable soluble salt form of the claimed polypeptide, such as hydrochloride salt, can be dissolved and administered in a pharmaceutical diluent such as pyrogen-free water (distilled), physiological saline or 5% glucose solution. A suitable insoluble form of the compound may be prepared and administered as a suspension in an aqueous base or a pharmaceutically acceptable oil base, e.g. an ester of a long chain fatty acid such as ethyl oleate.

According to a third aspect, the invention provides a method for screening for and/or diagnosing a neoplastic disease characterized by a change in lymph vessel endothelial cells. The method comprises obtaining a sample from an animal suspected of being in a disease state characterized by an increase in lymph vessel endothelial cells; exposing said a polypeptide comprising an amino acid sequence having at least a 90% identity with the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or a fragment thereof; washing said sample; and screening for said disease by detecting the presence, quantity or distribution of VEGF receptor-3 in said sample, where detection of an increase in expression of VEGF receptor-3 in or on lymph vessel endothelial cells in or around a potential neoplastic growth is indicative of a neoplastic disease.

It is clearly understood that for the purposes of this specification the term "sample" includes, but is not limited to, a tissue sample, blood, serum, plasma, urine, ascities

fluid or pleural effusion. Preferably the tissue is human tissue.

Polypeptides according to the invention may be labeled with a detectable label, and utilized to identify the VEGF receptor-3 *in situ*. The polypeptide may be covalently or non-covalently coupled to a suitable supermagnetic, paramagnetic, electron dense, ecogenic or radioactive agent for imaging. For use in diagnostic assays, radioactive or non-radioactive labels may be used. Examples of radioactive labels include a radioactive atom or group, such as ^{125}I or ^{32}P. Examples of non-radioactive labels include enzymatic labels, such as horseradish peroxidase or fluorimetric labels, such as fluorescein-5-isothiocyanate (FITC). Labeling may be direct or indirect, covalent or non-covalent.

It will be clearly understood that for the purposes of this specification the word "comprising" means "including but not limited to". The corresponding meaning applies to the word "comprises".

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of VEGF-D processing;

Figure 2 shows the results of Western blot analyis of human (H) and mouse (M) VEGF-DΔNΔC;

Figure 3 shows the results of the VEGFR-2 bioassay using human and mouse VEGF-DΔNΔC; and

Figure 4 shows the results of the VEGFR-3 bioassay using human and mouse VEGF-DΔNΔC.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Example 1 Expression and Purification of mouse VEGF-D

To test the receptor binding properties of mouse VEGF-D, a plasmid was constructed for expression of the VEGF Homology Domain (VHD) of mouse VEGF-D. A DNA fragment encoding amino acid residues 92 to 201 (SEQ ID NO:6) of full-length mouse VEGF-D2 (SEQ ID NO:4) was amplified by polymerase chain reaction (PCR) with *Pfu* DNA polymerase, using as template a plasmid comprising full-length mouse VEGF-D cDNA (SEQ ID NOs:1 or 3). The amplified DNA fragment, the correctness of which was confirmed by nucleotide sequencing, was then inserted into the expression vector pEFBOSSFLAG (a gift from Dr. Clare McFarlane at the Walter and Eliza Hall Institute for Medical Research (WEHI), Melbourne, Australia). The pEFBOSSFLAG vector contains DNA encoding the signal sequence for protein secretion from the murine interleukin-3 (IL-3) gene and the FLAG® octapeptide (IBI/Kodak). The FLAG® octapeptide can be recognized by commercially available antibodies such as the M2 monoclonal antibody (IBI/Kodak). The VEGF-D PCR fragment was inserted into the vector such that the IL-3 signal sequence was immediately upstream from the FLAG® octapeptide, which was in turn immediately upstream from the truncated VEGF-D sequence. All three sequences were in the same reading frame, so that translation of mRNA resulting from transfection of pEFBOSSFLAG-mouseVEGF-DΔNΔC into mammalian cells would give rise to a protein which would have the IL-3 signal sequence at its N-terminus, followed by the FLAG® octapeptide and the truncated VEGF-D sequence. Cleavage of the signal sequence and subsequent secretion of the protein from the cell would give rise to a VEGF-D polypeptide which is tagged with the FLAG® octapeptide adjacent to the N-terminus. This protein was designated

mouse VEGF-DΔNΔC. The expression cassette encoding the FLAG-tagged truncated VEGF-D construct was subcloned into the pAPEX-3 expression vector and then transiently expressed in 293EBNA-1 cells using Fugene (Boehringer Mannheim) mediated transfection. After seven days of incubation, the conditioned medium was collected (approximately 150 ml) and subjected to affinity chromatography using the M2 (anti-FLAG) beads (IBI/Kodak) according to the manufacturer.

SDS-PAGE and Immunoblotting

Mouse VEGF-DΔNΔC arising from affinity chromatography and purified human VEGF-DΔNΔC (for comparison purposes) were analyzed by Western blotting. About 50 ng of each protein was separately combined with SDS-PAGE sample buffer under reducing (2% β-mercaptoethanol) conditions, boiled and resolved by SDS-PAGE. The proteins were transferred to nitrocellulose and blotted with M2 antibody. As seen in Figure 2, both human and mouse VEGF-DΔNΔC subunits have the expected molecular weight of 22 kDa.

Example 2 Bioassay for mouse VEGF-DΔNΔC Binding to VEGF Receptors-2 and -3

The capacity of mouse VEGF-DΔNΔC to bind and cross-link VEGFR-2 and VEGFR-3 was tested in bioassays. Figures 3 and 4 shows the results of analysis of mouse VEGF-DΔNΔC protein using a VEGFR-2/-3 bioassay, respectively. The bioassay was performed using Ba/F3 cells which express a chimeric receptor consisting of the extracellular domain of mouse VEGFR-2 or human VEGFR-3 and the transmembrane and cytoplasmic domains of the mouse erythropoietin receptor (EpoR). These cell lines die in the absence of IL-3, unless they are supplied with ligands that cross-link the chimeric receptors. Cross-linking of the VEGFR/EpoR chimeric receptors induces

signaling from the EpoR cytoplasmic domains that stimulates cell survival and proliferation.

The cells were maintained in Dulbecco's Modified Eagle Medium(DMEM) containing 10% fetal bovine serum (FBS), 50 mM L-glutamine, 50μg/ml gentamicin and 10% of the Walter and Eliza Hall Institute of Medical Research (WEHI)-3D-conditioned medium as a source of interleukin-3 (IL-3). Cells expressing the VEGFR-2-EpoR or VEGFR-3-EpoR chimeric receptor were washed three times in phosphate buffered saline (PBS), and once in complete medium lacking IL-3. Cells (10^4) were aliquoted into 96-well microtiter plates containing dilutions of the test reagent or medium alone. Cells were incubated for 48 hours at 37°C in a humidified atmosphere of 5% CO_2. Cell proliferation was quantified by the addition of 1 μCi of ^{3}H-thymidine for four hours prior to harvesting. Incorporation of ^{3}H-thymidine was determined using a cell harvester and β-counting.

As mentioned above, activation of the chimeric receptor rescues the cells from their dependence on IL-3 and causes the cells to proliferate in the absence of IL-3. Human VEGF-DΔNΔC which is a ligand for both VEGFR-2 and VEGFR-3, stimulates growth of these cell lines in a specific and dose-dependent fashion (see Figures 3 and 4, respectively). Mouse VEGF-DΔNΔC was able to simulate growth of VEGFR-3/EpoR cell line in a specific and dose-dependent fashion, but had no significant effect on the VEGFR-2/EpoR cell line even at a concentration as high as 4 μg/ml (see Figures 4 and 3, respectively). Assays were carried out in duplicate and error bars denote a standard deviation of +/- 1.0. This unexpected finding demonstrates that mouse VEGF-DΔNΔC is not an activating ligand for VEGFR-2. Note that the VEGFR-2 extracellular domain in the chimeric VEGFR-2/EpoR receptor expressed in the Ba/F3-VEGFR-2-EpoR cell line was derived

from mouse VEGFR-2. Therefore the inability of mouse VEGF-DΔNΔC to induce survival and proliferation of these cells was not due to a species difference between this ligand and the extracellular domain of the chimeric receptor.

The foregoing description and examples have been set forth merely to illustrate the invention and are not intended to be limiting. Since modifications of the disclosed embodiments incorporating the spirit and substance of the invention may occur to persons skilled in the art, the invention should be construed broadly to include all variations falling within the scope of the appended claims and equivalents thereof.

WHAT IS CLAIMED IS:

1. A method for stimulating proliferation and/or maintaining of only lymph vessel endothelial cells, in an animal in need of such treatment, comprising:

administering to said cells an effective amount of a composition comprising a polypeptide having at least a 90% sequence identity with the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or a fragment thereof which has the ability to only stimulate one or more of lymphatic vessel endothelial cells to proliferate, differentiate, migrate or survive.

2. The method of Claim 1, wherein the polypeptide has at least a 95% sequence identity with the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or a fragment thereof.

3. A method for activating only a VEGF receptor-3, comprising:

administering to a cell bearing said receptor an effective amount of a composition comprising a polypeptide having at least 90% sequence identity with the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or a fragment thereof.

4. The method of Claim 3, wherein the polypeptide has a 95% sequence identity with the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or a fragment thereof.

5. The method of Claim 3, wherein the method is carried out *in vivo*.

6. The method of Claim 3, wherein the method is carried out *in vitro*.

7. A method for screening for a neoplastic disease in an animal suspected of being in a neoplastic disease state characterized by an increase in lymph vessel endothelial cells, comprising:

obtaining a sample from said animal;

exposing said sample to a polypeptide comprising an amino acid sequence having at least a 90% identity with the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or a fragment thereof;

washing said sample; and

screening for said disease by detecting the presence, quantity or distribution of VEGF receptor-3 in said sample, where detection of an increase in expression of VEGF receptor-3 in or on lymph vessel endothelial cells in or around a potential neoplastic growth is indicative of a neoplastic disease.

8. A method of identifying only lymph vessel cells, comprising:

obtaining a sample from an organism;

exposing said sample to a polypeptide comprising an amino acid sequence having at least a 90% identity with the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or a fragment thereof;

washing said sample; and

detecting binding of said polypeptide to said receptor by any suitable means.

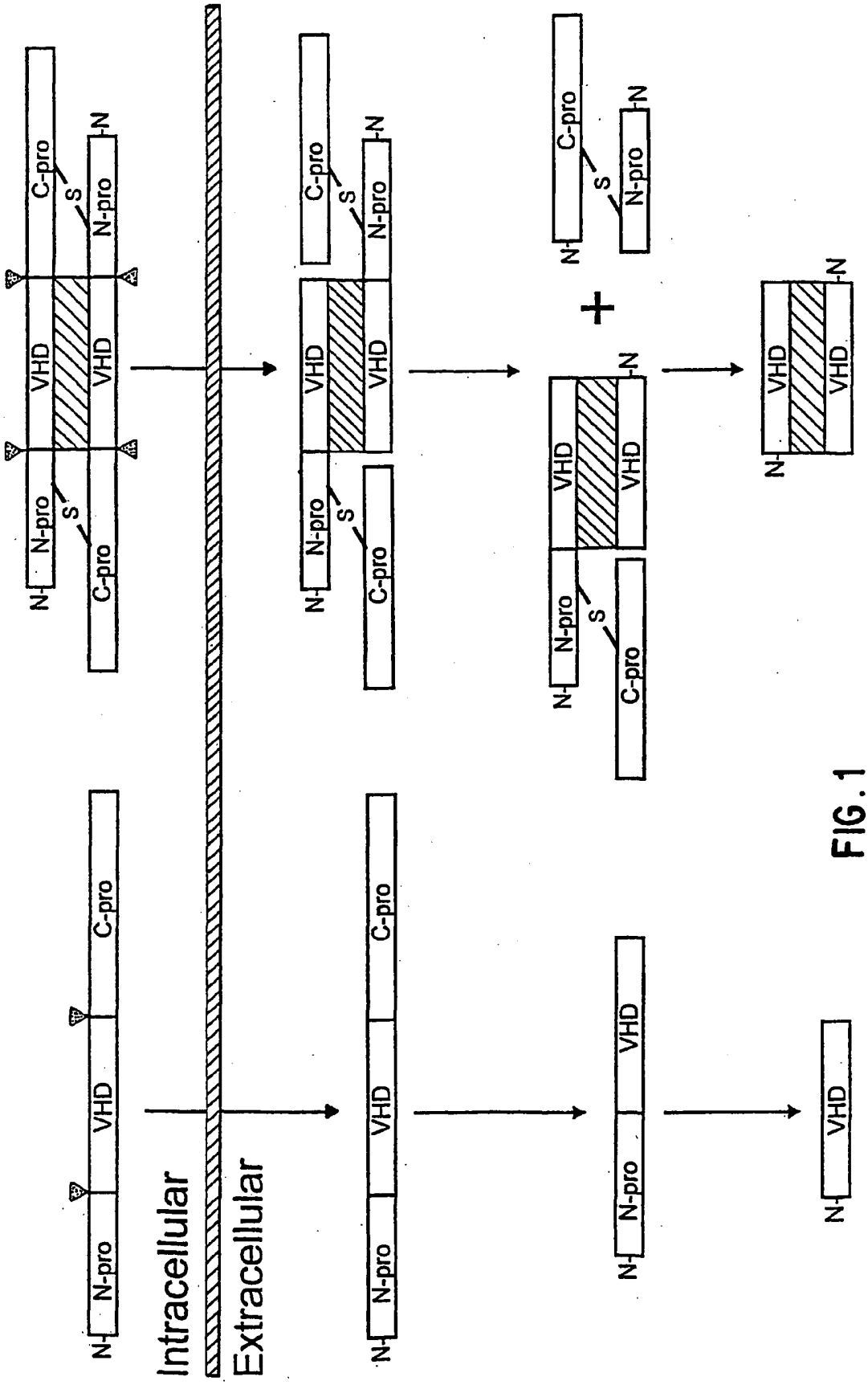
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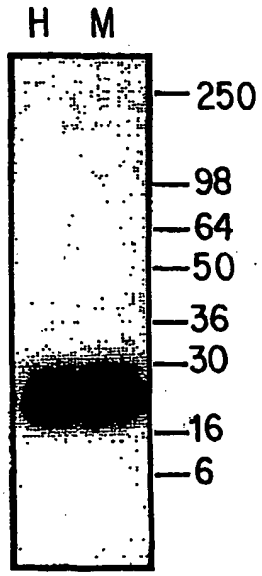
FIG. 1



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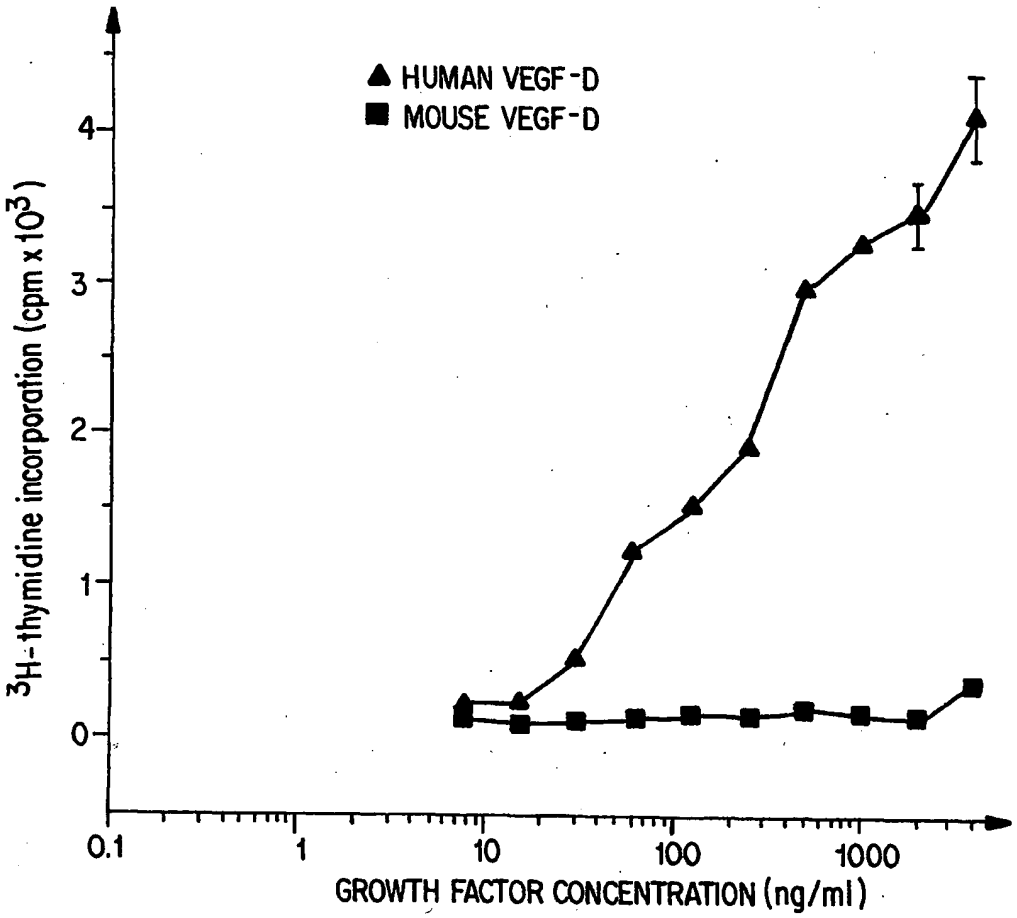
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MOUSE VEGF-D
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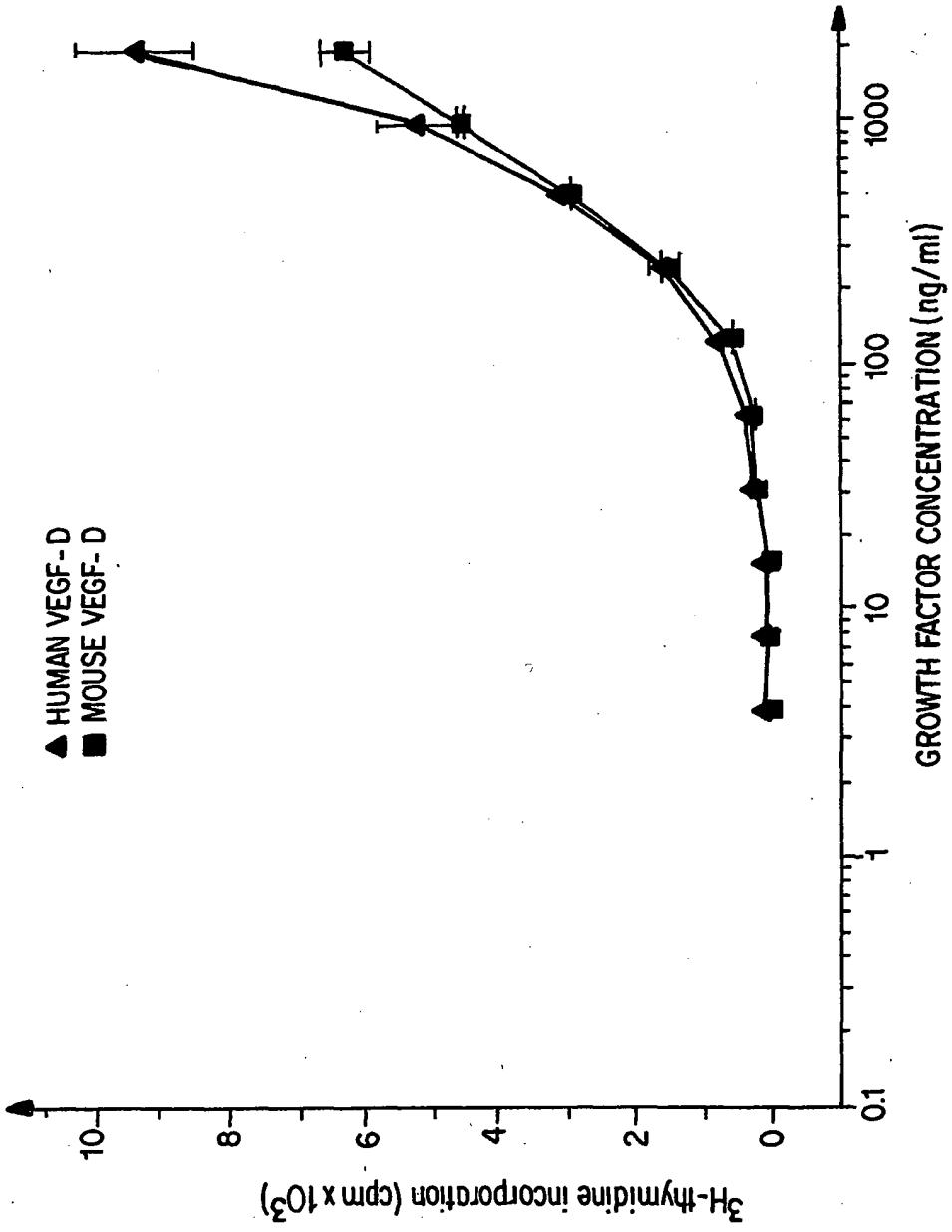
GROWTH FACTOR CONCENTRATION (ng/ml)

FIG.3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MOUSE VEGF-D
GROWTH FACTOR CONCENTRATION (ng/ml)
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4
6
8
10

FIG. 4

SEQUENCE LISTING

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STACKER, Steven A

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220                 225                 230

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         35                  40                  45

Ser Leu Glu Glu Leu Leu Gln Ile Ala His Ser Glu Asp Trp Lys Leu
     50                  55                  60

Trp Arg Cys Arg Leu Lys Leu Lys Ser Leu Ala Ser Met Asp Ser Arg
 65                  70                  75                  80

Ser Ala Ser His Arg Ser Thr Arg Phe Ala Ala Thr Phe Tyr Asp Thr
                 85                  90                  95

Glu Thr Leu Lys Val Ile Asp Glu Glu Trp Gln Arg Thr Gln Cys Ser
            100                 105                 110

Pro Arg Glu Thr Cys Val Glu Val Ala Ser Glu Leu Gly Lys Thr Thr
        115                 120                 125

Asn Thr Phe Phe Lys Pro Pro Cys Val Asn Val Phe Arg Cys Gly Gly
    130                 135                 140
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```
Cys Cys Asn Glu Glu Gly Val Met Cys Met Asn Thr Ser Thr Ser Tyr
145                 150                 155                 160

Ile Ser Lys Gln Leu Phe Glu Ile Ser Val Pro Leu Thr Ser Val Pro
                165                 170                 175

Glu Leu Val Pro Val Lys Ile Ala Asn His Thr Gly Cys Lys Cys Leu
            180                 185                 190

Pro Thr Gly Pro Arg His Pro Tyr Ser Ile Ile Arg Arg Ser Ile Gln
        195                 200                 205

Thr Pro Glu Glu Asp Glu Cys Pro His Ser Lys Lys Leu Cys Pro Ile
    210                 215                 220

Asp Met Leu Trp Asp Asn Thr Lys Cys Lys Cys Val Leu Gln Asp Glu
225                 230                 235                 240

Thr Pro Leu Pro Gly Thr Glu Asp His Ser Tyr Leu Gln Glu Pro Thr
                245                 250                 255

Leu Cys Gly Pro His Met Thr Phe Asp Glu Asp Arg Cys Glu Cys Val
            260                 265                 270

Cys Lys Ala Pro Cys Pro Gly Asp Leu Ile Gln His Pro Glu Asn Cys
        275                 280                 285

Ser Cys Phe Glu Cys Lys Glu Ser Leu Glu Ser Cys Cys Gln Lys His
    290                 295                 300

Lys Ile Phe His Pro Asp Thr Cys Arg Ser Met Val Phe Ser Leu Ser
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Pro
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Arg Thr Gln Cys Ser Pro Arg Glu Thr Cys Val Glu Val Ala Ser Glu
             20                  25                  30

Leu Gly Lys Thr Thr Asn Thr Phe Phe Lys Pro Pro Cys Val Asn Val
         35                  40                  45

Phe Arg Cys Gly Gly Cys Cys Asn Glu Glu Gly Val Met Cys Met Asn
     50                  55                  60

Thr Ser Thr Ser Tyr Ile Ser Lys Gln Leu Phe Glu Ile Ser Val Pro
 65                  70                  75                  80

Leu Thr Ser Val Pro Glu Leu Val Pro Val Lys Ile Ala Asn His Thr
                 85                  90                  95

Gly Cys Lys Cys Leu Pro Thr Gly Pro Arg His Pro Tyr Ser
            100                 105                 110
```